Biochemical aspects of xylem differentiation in plant tissue culture

Thesis

BIOCHEMICAL ASPECTS OF XYLEM DIFFERENTIATION IN PLANT TISSUE CULTURE

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Presented to The Open University in December, 1982 in fulfillment of the requirements for the Degree of Doctor of Philosophy.

Date of submission: 1.12.82

Date of award: 26.1.83

Dedicated to my wife, Margaret, and sons,

John and Tom.

ABSTRACT

Plant cytodifferentiation is discussed in general terms with special reference to the 'model system' of xylem differentiation. Previous findings relating to polysaccharide, nucleic acid and lignin synthesis are reviewed in the introduction, while detailed reviews relating to protein synthesis, protein covalent modification and plant hormone binding are located under the appropriate chapter heading.

A list of criteria necessary for the use of plant tissue cultures in detailed biochemical analysis of xylogenesis is discussed with reference to other reported xylogenic cultures, none of which fully meet these requirements. A previously utilised Jerusalem artichoke (*Helianthus tuberosus*) tuber explant system has been modified and now appears to meet the above criteria. This two step culture allows for the separation of infected explants prior to their further culture on a xylogenic medium or a control medium (on which the explants grow at the same rate as those on the xylogenic medium but without a significant formation of xylem elements). Using this culture regime it should be possible to separate the biochemical events associated with the initial excision wound response of the artichoke tissue, tissue growth and xylem differentiation.

An investigation into a particulate and cytosol 2,4-D specific binding protein showed fluctuations in tissue concentration throughout the culture periods and while these fluctuations did not correlate with the xylogenic

process they did appear to coincide with a wound or subculture response of the cultured artichoke tissues.

Utilising the two step artichoke culture system, protein synthesis was analysed by one and two dimensional gel electrophoresis, following radiolabelled amino acid incorporation. Investigations of protein covalent modification (namely glycosylation and phosphorylation) using radiolabelled precursor incorporation, one dimensional gel electrophoresis and autoradiography/fluorography have also been undertaken. Several fluctuations were noted in the syntheses, which may be of significance in the xylogenic process, the most marked alterations being in the phosphorylation of cytosol located polypeptides which coincided with the peak rate of xylem differentiation. To complement the phosphorylation studies, protein kinase activity was extracted from cultured artichoke tissue, and found to be modulated by the *in vitro* addition of kinetin and cAMP (which appears to act as a cytokinin) and by 2,4-D. This allows for the possibility of a direct hormonal control of xylem differentiation in plant tissues, acting, at least in part, through the modulation of protein kinase activity and the phosphorylation of possible key proteins associated with the cyto-differentiation process.

Acknowledgements

My gratitude is expressed towards various people for help given throughout my postgraduate studies and in the writing of this thesis.

Principally to my supervisor, Lindsay Haddon.

To members of the Plant Research Group, including: Irene Ridge, Mary Bell, Mike Malone, Doug Wilson, Ivan Amarasinghe and Jonathan Silvertown.

To Professor Steven Rose and other members of the Biology Department, who have shown interest in my research, especially Sean Murphy and John Rudge.

To the typist, Lyn Cambourne-Paynter

To the Graphics and Photography Department

A paper relating to the chapter on xylogenic cultures has been published, 'An improved technique for the culture of Jerusalem Artichoke (Helianthus tuberosus L.) explants for use in the study of xylem differentiation'. Plant Cell Reports (1982) 1: 229-231.

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ABBREVIATIONS

BAP	Benzylamino purine
BHT	Butylated hydroxytoluene
CAMP	Adenosine 3':5'-cyclic monophosphate
2,4-D	2,4-Dichlorophenoxyacetic acid
2D-SDS-PAGE	Two dimensional-sodium dodecyl sulphate-polyacrylamide gel electrophoresis
EDTA	Ethylenediaminetetra acetic acid
IAA	Indoleacetic acid
IEF	Isoelectric focusing
NAA	Napthaleneacetic acid
PMSF	Phenylmethylsulphonylfluoride
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-Tetramethylethylenediamine

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CHAPTER ONE

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INTRODUCTION

The limited extent of the understanding in plant and animal developmental biology maybe considered as rudimentary when compared to other aspects of biological knowledge. This is not too surprising when one considers the undoubted complexity and subtlety of cellular differentiation, including the various and varying control mechanisms encompassed within this process (Shininger 1979). None the less investigations into cytodifferentiation continue and it is still true to say, even given the advances made in this field, that the molecular minutiae of cytodifferentiation remain an elusive quest of modern biology.

Multicellular eukaryotic morphogenesis is composed of two inter-related phenomena; cellular differentiation (a qualitative process) and cellular growth (a quantitative process). Differentiation not only occurs at the cellular level but also moulds cellular aggregates, in the organism, as tissues and organs. Cytodifferentiation is normally, and by necessity, a very ordered process, in which one stage follows another within a constraining process. This indicates tight control and relates to the central problem, which is; how do cells of a common lineage diverge along alternative pathways? In other words, how does cell heterogeneity originate? In plants all cells originate from a meristematic region, within either the apices or at certain lateral locations, and from these relatively simple regions of rapid division develops the full spectrum of cell types of which the whole plant is composed, with all its complexity of form and function.

There is a general assumption that the control of cytodifferentiation must utilise known biological phenomena, although many of the details are either unknown or not completely understood. Utilising the available knowledge together with the various hypothetical models for cytodifferentiation, it is possible to make several general statements regarding cellular differentiation. Firstly, the process must be genetically controlled, as Roberts (1976) points out, there is no such thing as an undifferentiated cell without a genetic programme. Terminal cells are products of precursor cells with unique genetic programmes. Secondly, there must be some element of intercellular communication within differentiating plant tissues. Communication, presumably of a chemical nature, allows for both a positional awareness of the individual differentiating cell and a coordination of the development within the tissue. Both aspects are vital in the overall morphogenetic process. Finally it is self evident that structural and functional differences, between cells originating from the same growing point, must have a molecular basis. It is probably the ultimate aim of developmental biologists to understand cytodifferentiation in molecular terms.

It maybe useful to relate the terms used by Roberts (1976) when discussing differentiation. 'Cellular determination' is the process undergone when a cell becomes restricted to a new and special pathway of development. The 'point of determination' is the transient period during which cytodifferentiation is initiated. This process is reversible and this fact is utilised in plant tissue cultures, in the sense that a commonly cultured tissue such as tuber parenchymatous tissue i.e. differentiated cells, can generate new cells, such as xylem elements, under appropriate conditions. The 'cytodifferentiation sequence' encompasses the point of determination and all stages up to the fully mature cells.

Tracheophyta (vascular plants) are characterised by associated specialised conducting tissues; xylem and phloem, an adaptation to terrestrial existence. The term xylem refers to a plant tissue composed of parenchyma, sclerenchyma, and characteristic tracheary elements (vessel elements or tracheids) which are arranged in specific patterns in the various plant organs. Xylem elements are thick walled cylindrical cells which are filled with water on maturity forming a continuous tubular system throughout the plant. The elements have recognisable secondary wall sculpturing of four major types; helical, spiral, reticulate and pitted wall formations.

Xylem tissues in whole plants originate from the meristematic regions and may also originate as a wound response in a partially severed region of the plant. There is some evidence that, while xylem are formed in the above differing locations, the basic biochemistry of the differentiation process is very similar, while differing only in certain details.

The xylem element is probably the most extensively studied plant cell type, in terms of its induction in whole plants and tissue culture, and the cell biochemistry associated with its formation. It has been considered as a "paradigm" (Torrey et al. 1971) or a "model system" (Roberts 1976) of plant cytodifferentiation. Xylem differentiation has been reviewed by Bryant (1976), Shininger (1979), Phillips (1980) and most extensively by Roberts (1976). The choice of xylem as a model cell type, in the majority of investigations, has been determined on pragmatic grounds. It is ubiquitous to cultured plant tissues, it is easily identified and quantified and it has a clear cytodifferentiation sequence (Torrey et al. 1971). Of equal importance, there have recently been published details of several culture systems which approach the ideal synchronous production of large numbers (as a proportion of the total tissue cell number) of xylem elements.

The availability of such an ideal system lends xylem to a detailed study of plant cytodifferentiation.

Xylem element formation has been investigated using whole plants, wounded regions of whole plants, plant parts, and increasingly, various culture systems. While many *in vivo* studies have provided much useful information about plant cell differentiation, the use of culture methods for this study has been expanding over recent years. The reasons for the change to tissue culture systems are several fold (Phillips 1980). Firstly, in whole plants the proportion of cells undergoing xylogenesis at any one time is low, and while the region of differentiation is precisely localised in the root or shoot, and is excellent for certain histological studies, it is insufficient for detailed studies of the cytodifferentiation sequence at the biochemical level. Secondly, culture techniques tend to use simple starting plant material, usually parenchymatous tissue, for the induction of xylem differentiation rather than whole or parts of plants, which are composed of a much more complex mixture of interacting cells and tissues. Thirdly, there is a greater degree of experimental control using plant culture techniques than would be available when observing or analysing xylem formation in whole plants.

The majority of tissue cultures use differentiated parenchymatous tissue as a starting point. The tissue is then induced to undergo dedifferentiation followed by a redifferentiation of certain of the cells into xylem elements. As Phillips (1980) states, it should be possible, using plant culture techniques, to develop a model system with the aim of unravelling the control factors operating at the point of determination and ultimately the changes in gene expression that lead to cytodifferentiation.

Certain doubts maybe raised with regard to xylem differentiation initiated in cultured plant tissues in comparison to normal intact plant xylogenesis. As already stated most studies utilise parenchymatous tissues, which dedifferentiate prior to forming xylem elements, whereas tracheary elements usually arise, in normal development, from a procambial region. Xylem elements formed in cultured tissues invariably have a reticulate secondary wall pattern whereas in whole plants the normal range of secondary wall formations are observed. While the validity of xylem induction studies using culture techniques is open to question, it would indeed be very surprising if the biochemical events closely associated with xylogenesis in the intact plant and in cultured tissues are not very similar if not indeed identical.

The Ontogenetic Stages of Tracheary Element Differentiation

One of the bonuses of studying xylem elements as a model differentiating system is that the developmental sequence is so precise. This should, therefore, allow a detailed analysis of the various cellular events occurring at each developmental stage. Listed below are the ontogenetic stages of tracheary element formation. Roberts (1976) considers the ontogeny of tracheary element formation to have four stages. During the first stage a transient competence to undergo cytodifferentiation is followed by DNA synthesis and/or cell division. It is thought that cellular determination probably involves a particular period of the cell cycle and it is known that protein synthesis is a requirement during this stage. The differentiating cell then expands. This involves the laying down of primary wall material, which is synthesised in the golgi apparatus and transported to the plasma membrane. Microtubules are thought to be of significance in the transport and orientation of wall material. Continued protein synthesis and DNA replication (including endoreduplication and gene amplification)

occur also. Thirdly, cellular enlargement continues with the polysaccharide fraction being synthesised changing to secondary wall characteristics, hemicellulose and cellulose predominating. Lignin precursor pools are formed and key polyphenolic associated enzymes are activated. Deposition of lignin into the cell wall occurs. Finally, degradative enzymes are released into the cytoplasm, possibly from lysosomes, causing organelles and membranes to be lysed. The degraded protoplast is removed. Enzymatic degradation of the non-lignified wall areas occurs to allow water flow through the end walls and laterally.

Biochemical Aspects of Xylem Element Formation

Biochemical research into xylogenesis can be grouped into the following categories:

(a) Cell wall biochemistry (wall composition, synthesis and lignification) and xylogenesis.

(b) Nucleic Acid biochemistry, the cell cycle and xylogenesis.

(c) Protein synthesis and xylogenesis.

This list of categories also reflects the order of the depth of knowledge for each, as it relates to the xylogenic process. The majority of the biochemical results related in this report will refer to the role protein synthesis plays in xylem element formation, together with the development of a modified and improved xylogenic culture and the study of a novel plant hormone binding protein in the cultured tissue. To be able to appreciate the relevance of these findings in this report it is necessary to discuss the previous findings in carbohydrate and nucleic acid biochemistry as they relate to xylem formation to give a broader view of the research undertaken.

Carbohydrate Associated Biochemistry

Many of the details regarding cell wall associated polysaccharide synthesis and its metabolic control during plant cell differentiation, including xylem differentiation, have been reviewed by Northcote (1969). The research into cell wall biochemistry during plant cytodifferentiation has been approached on three levels. Firstly an analysis of the various polysaccharide classes present in the cell wall and their individual sugar compositions during differentiation together with, and secondly, a quantification and evaluation of the enzyme activity changes associated with the cell wall during differentiation. Thirdly, an attempt has been made to determine the sub-cellular site(s) of polysaccharide synthesis during cellular differentiation.

In terms of cell wall biochemistry, differentiation can be followed sequentially from the newly synthesised cell plate of the dividing cell (later to become the middle lamella of the mature cell) to the primary wall of the elongating cell and, if appropriate to the differentiated cell type, the secondary wall. Xylem elements characteristically have a thick and finely sculptured secondary wall. An analysis of the sugar composition of the wall in the differentiating cell naturally reflects the maturation process. While the cell plate is mainly composed of pectins, the primary cell wall, which is laid down on the inner face of the plate, is composed of pectins, hemicelluloses and an increasing amount of cellulose as the primary wall builds up. A glycoprotein, 'extensin' is also present in the primary wall and is thought to have a structural role in the plant cell wall, possibly having some element of control in cell elongation (Lamport 1977). The secondary wall differs in composition with no pectin or extensin being deposited and the amount of hemicellulose decreasing while cellulose increases until it predominates by completion of the secondary wall.

Autoradiography and pulse chase experiments have been utilised to determine the sites of polysaccharide synthesis and the intracellular routes of transport from these sites to deposition in the wall. The golgi apparatus has been shown to be of importance in pectin and hemicellulose synthesis, with vesicles being the major source of transport to the cell wall. Cellulose is thought to be synthesised directly at the plasma membrane by synthetase complexes (e.g. Giddings et al. 1980). Microtubules appear to be important in the process of wall synthesis, possibly channelling polysaccharide-containing vesicles to the site of deposition or in the orientation of the synthetase particles at the membrane. Whatever the manner in which microtubules do exert their influence, they are known to be important in secondary wall pattern formation being located under the xylem element secondary wall banding, the orientation of which is an important factor in the strength giving properties of the secondary wall (Heath 1974).

Throughout the changes in the cell wall during cellular differentiation there is a gradual alteration in the polysaccharide composition and the polysaccharide constituent sugar monomers. These alterations must be controlled by "sugar associated enzymes", the modulation either occurring at the coarse level, i.e. by controlling the amount of precursor molecules available, or more finely by the control of the polysaccharide synthetase enzymes. Both types of polysaccharide compositional control have been noted. A coarse control of pectin and hemicellulose precursors has been noted in the differentiating xylem of poplar trees (Dallesandro and Northcote 1977a) and pine and fir trees (Dallesandro and Northcote 1977b). Bolwell and Northcote (1981) observed the finer control in bean hypocotyl and callus culture, for example the decrease in pectin synthesis correlating with the reduction in arabinan synthetase activity. Dallesandro and Northcote (1981 a and b) also found a large increase in xylan synthetase activity

associated with xylem secondary wall synthesis in sycamore and poplar tree vascular cambium. In summary the activity of the various polysaccharide associated enzymes reflects the development of the xylem wall. This requires the synthesis or activation of the requisite enzymes involved and means that the changes known to occur should be able to be analysed using the various chemical or physical/chemical techniques available.

Lignification

The lignification process can be considered as a terminating differentiation sequence in xylem element formation, however it has no bearing on the early events of xylem initiation but acts as a conclusion to the previous components of the cytodifferentiation sequence. Lignin is an irregular polymer of hydroxylated and methylated phenylpropanoid residues. It is present in xylem cell walls and also in other cell walls such as that of sclerenchyma, where it provides the structural function evident in xylem elements. Lignin impregnates all layers of the xylem wall and together with the polysaccharides present confers rigidity on the cell wall and protection to parts of the wall during the autolytic stage of xylem maturation (Barrett 1979).

The lignification of xylem elements has been studied by Hepler et al. (1970 and 1972) who found a similarity between the localisation of peroxidase and lignin in *Coleus* wound vessel walls, suggesting that the enzymes associated with lignification are localised in regions of the wall undergoing this process.

The precursor for the phenylpropanoid alcohols is phenylalanine and the enzyme phenylalanine ammonia lyase (PAL) plays a key role in the channelling of this substrate into the phenylpropanoid synthetic pathway. In view

of the central role of PAL, many attempts have been made to designate PAL actively as a biochemical marker for xylem differentiation. PAL activity has been correlated with xylem formation (Rubery and Fosket 1969, Haddon and Northcote 1975 and 1976 and, most recently, Fukuda and Komamine 1982), however Minocha and Halperin (1976) using cultured artichoke explants only showed a limited correlation, and pointed out complications of the attempts to find marker enzymes. In this tissue not only are the xylem elements lignified but parenchymatous tissue also, thereby making it impossible to designate to PAL the marker function anticipated.

Nucleic Acid Biochemistry, the Cell Cycle and Xylogenesis

The close control of cellular differentiation indicates that the genome plays at least some part in the process. Investigators have undertaken studies into both nucleic acid synthesis and the cell cycle as they relate to the differentiation of xylem elements. The research falls into two inter-related groups; firstly DNA synthesis, including endoreduplication and gene amplification studies, and secondly, studies on the cell cycle and it's relationship to xylogenesis.

Early studies, utilising both intact plants and plant tissue cultures, indicated that DNA synthesis is a requirement for xylem differentiation, which also has a close correlation with the cell cycle, principally cellular division.

Polyploidy of determined precursor xylem elements was considered to be of importance (Phillips and Torrey 1973) in that they suggested a cyto-differentiation sequence which included a hormonal stimulation of endoreduplication followed by polyploidal mitosis and terminal cellular differentiation. Differentiating xylem elements, in cultured pea root cortical

explants, were studied, in which the elements still retained their nuclei, even though the secondary wall had been formed, prior to the autocatalytic sequence. While the differentiating nuclei contained 2C, 4C, 8C and 16C DNA it did not differ significantly from the ploidy levels of non-differentiating cells, thereby they concluded that the ploidy level was independent of the differentiating process. Polyploidy, as a trigger for xylem element differentiation, has since been discounted when it was determined that artichoke differentiating cells remain exclusively at the 2C level (Dodds and Phillips 1977).

A related question is that of whether DNA synthesis and/or mitosis are related to or required for xylem formation. Fosket (1968) showed a requirement for DNA synthesis in *Coleus*. Inhibitors, such as fluorodeoxyuridine (FUdR) have been used extensively in these studies and been shown to prevent cell division and xylogenesis in pea (Shininger 1975), and artichoke (Phillips 1980). However, contrary to this, Turgeon (1975), studying wound vessel member differentiation in cultured explants of lettuce pith, observed xylem element formation without DNA synthesis, mitosis or cell division. This finding has been repeated by Torrey (1975), Fukuda and Komamine (1980) and Dodds (1980), all of whom witnessed xylem differentiation without intervening cell division. It was therefore concluded that cell division was not an absolute prerequisite for xylem formation.

Polyploidy i.e. chromosomal replication without cytokinesis, can be considered as a developmental regime for the gross amplification of the genome in much the same way that gene amplification is a more selective approach to the same solution, that of increasing available gene products. The root of *Allium cepa* has proved to be very useful in the study of gene amplification during xylem development. Avanzi et al. (1973) utilised a

tritiated rRNA cytological hybridisation technique to demonstrate a six fold increase in the cistrons for rRNA in root regions undergoing metaxylem formation. Further to this Innocenti (1975) showed that during metaxylem formation there are cyclical changes in the histone/DNA ratio within a developmental sequence which includes endoreduplication followed by a conspicuous extrasynthesis of DNA in the nucleolus associated chromatin, which is then released. As well as the above fluctuations, Durante et al. (1979) also found changes in "other DNA families" during metaxylem differentiation in onion roots. The use of several different DNA inhibitors by Fukuda and Komamine (1981) in *Zinnia* cultures indicated that while the whole genome does not need to be replicated, for xylem elements to be formed, there is a requirement for some DNA synthesis. In summary the inhibitor studies signify that an element of DNA synthesis is required for xylogenesis with different plant species attaining this via different routes including; endo-reduplication, replication and specific gene amplification.

A close relationship exists between cytodifferentiation and the cell cycle in eukaryotic organisms (Fosket 1968), indicating that the molecular mechanisms governing differentiation may be regulated, to some extent, by DNA replication or mitosis (given the reservations already discussed).

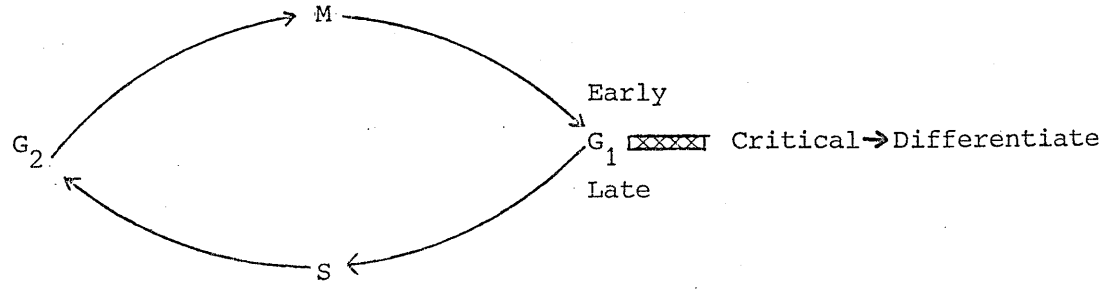
In whole plant cellular differentiation it is not clear if a cell becomes committed to a differentiation pathway during meristematic activity or at a later stage (Roberts 1976). Siebers (1971) has proposed a model for interfascicular located cells in which the cells are programmed for cytodifferentiation during an earlier meristematic period, functioning in a predifferentiated state until later, when they are triggered to differentiate, possibly by hormonal action. In this model, therefore, determination does not necessarily mean immediate cellular transformation.

It has been postulated that some period of the cell cycle is involved in the, initiation of cytodifferentiation. This may include the *de novo* appearance of protein(s) which could be involved directly as an enzyme in the differentiation process, or as an activator of the genome or even be involved in the turnover of plant hormones required for differentiation to occur. To this end several studies have been undertaken to map enzyme activities during the cell cycles of synchronous cultures such as cultured Jerusalem artichoke explants (Yeoman and Aitchison 1973), which expresses synchronous division at least in the first three days of culture.

Malawer and Phillips (1979), using tritiated thymidine autoradiography, studied the periods of DNA synthesis in artichoke cells destined to differentiate into xylem elements. They determined that the tuber parenchymatous cells go through three rounds of DNA synthesis and mitosis prior to differentiating into xylem at the G_1 stage of the cell cycle. In addition, Phillips (1981) estimated that the first visible signs of element differentiation occurred between 7 and 10 hours after the last mitosis.

The *Zinnia* mesophyll cell suspension culture also shows partial synchrony of DNA synthesis, mitosis and xylem differentiation. Utilising similar techniques to that used in the artichoke studies, Fukuda and Komamine (1981) concluded that the initiation of xylogenesis in cultured *Zinnia* cells again occurs at the G_1 phase. However cytodifferentiation in the mesophyll cells is independent of the cell cycle, with both being able to proceed concurrently. The artichoke and *Zinnia* cells therefore demonstrate a difference in their ability to undergo direct transformation, without an intervening cell cycle, as in *Zinnia*, as opposed to the need to undergo several cell cycles prior to xylogenesis, as in artichoke. Dodds (1981), in an attempt to unify these differences has proposed a model in

which the G_1 phase is split into an early and a late G_1 phase with an intervening period, which Dodds calls a "critical events zone", in which the differentiation step is taken.



Those cells 'arrested' in the early G_1 phase can simply be transformed directly into xylem elements e.g. *Zinnia*, but those arrested in late G_1 must undergo DNA synthesis before cycling back to the critical zone to differentiate e.g. artichoke.

Plant Hormone Receptors and Xylogenesis

A good deal of research and an ever increasing acquisition of data relating to putative plant hormone (growth substance) 'receptors' has grown over recent years. (Reviewed by Kende and Gardner 1976). The search for plant hormone receptors has, in many ways, been an attempt to emulate the advances made in the study of animal systems. However while in the animal studies, certain *in vivo* hormone binding has been allocated a function and the primary responses of the hormone receptor complex formation have been elucidated and repeated *in vitro*, plant hormone binding has yet to be designated a definite function.

The term 'receptor' implies the hypothesis that a plant hormone should control the activity and/or the spectrum of gene products, in terms of hormone recognition by specific receptors, whose occupancy then initiates

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CHAPTER TWO

a chain of events which produce the observed hormonal response. Given the defining criteria for use of the term 'receptor', there have been no true receptors isolated from plant tissues and no function has been determined for the plant hormonal specific binding found in these tissues. However, there are indications that plant hormone specific binding is found in the various subcellular fractions of plant tissues and there exists the possibility that there is a function for this binding in plants as in animals, but to cross the phylogenetic boundary readily maybe too simplistic an approach. While biological general principles and many biochemical details hold true for both plants and animals, multicellular plants are not "green animals", hence there must also be basic differences. An example of this is the large number of animal hormones which have a tendancy to initiate specific responses when compared to the much fewer plant growth substances, each having a multitude of effects. This difference may indicate that there is a genuine dissimilarity in plants and animals as to the primary site of hormone action (Trewavas 1979).

Auxin physiology has been studied, with respect to its specific binding, in an attempt to correlate the binding with: (i) the stimulation of cell enlargement in shoot tissue and the "acid growth hypothesis", (ii) the polar transport of auxins and (iii) the ability to modulate or trigger macromolecular biosynthesis (reviewed by Rubery 1981). Other plant hormones have been studied, but to a much more limited extent.

The tissue concentration of auxin specific binding factors has been shown to change in cultured plant tissues, including artichoke tuber tissue (Trewavas 1981), over the culture period but there has been no attempt to correlate changes in specific plant hormone binding within a differentiating process. Combinations of plant hormones as well as specific levels of particular hormones e.g. auxins, have been demonstrated to be significant

in the induction or control of xylem element formation. It was decided to undertake an analysis of auxin binding protein(s) in cultured artichoke tissues with a view to determining a possible correlation between this specific binding and xylem differentiation.

Protein Synthesis and Xylem Differentiation

The differentiation of a xylem element manifests itself as a controlled sequence of cytological events, terminating in a finely sculptured, cytoplasm-free, functioning element. Given this closely controlled cytodifferentiation sequence, one might expect the induction or activation of specific enzymes or structural proteins to precede such a formidable cellular transformation. One of the simplest definitions of cytodifferentiation (Jacob and Monod 1963) states that "two cells are differentiated with respect to each other if, while containing the same genome, the pattern of proteins synthesised by each is different". The study of proteins contained within a differentiating cell is an obvious direction of investigation and should provide valuable information with regard to the control of differentiation, hopefully leading to the identification of key modulations associated with the cytodifferentiation sequence. Relatively little research has been reported on this aspect of xylogenesis, while that work which has been reported concerns itself mainly with the gross accumulation or incorporation of radioactively labelled amino acids into differentiating tissues. No detailed analysis of the proteins present or synthesised during xylem differentiation has been published.

It has been stated that the control of biological development and differentiation will be dependent, to a greater or lesser extent, on the turnover of certain key protein constituents. The overall turnover of an enzyme or structural protein is a balance between protein synthesis

(transcription and translation) and degradation. A further level of control for certain proteins is that of post-translational modification, a covalent modification in which a specific chemical group is covalently attached to the precursor protein to produce a modulated final product. Protein covalent modifications include; methylation, acetylation, thiolation, ribosylation, glycosylation, adenylation and phosphorylation. By far the majority of present day knowledge relates to the phosphorylation of proteins.

Glycoproteins are important cellular constituents, being located in both the cytosol and the membranous fractions. The biochemistry and, often speculative, functions of glycoproteins have been reviewed by Brown and Kimmins (1977) and Sharon and Lis (1979). Animal glycoproteins have been studied in more detail, when compared to plant proteins, with both sources demonstrating many common features including examples of enzymes, lectins and structural proteins. The retardation of research into plant glycoproteins has in many respects been as a result of the difficulties encountered in extracting the glycoproteins intact.

The function of the carbohydrate moiety within the glycoprotein is far from understood, although it is known that the *in vitro* properties of proteins are modified on glycosylation e.g. susceptibility to denaturation and proteolysis. Brown and Kimmins (1977) suggested that the carbohydrate group increases the number of surface interactions with water and cellular structures, thereby acting as a protection to the protein structure. The carbohydrate groups may also enhance the stability of enzymes or contribute towards their interactions with membranes or within a multienzyme complex. However the majority of roles attributed to glycoproteins, within animals and plants, are those of cellular and molecular recognition.

Protein phosphorylation is recognised as a major mechanism by which intracellular events respond to external physiological stimulii. The

classic example of such a process is found in animal systems where, for instance, the hormone adrenaline causes the breakdown of intracellular glycogen through a complex interaction of enzymes, including; adenyl cyclase, phosphorylase kinase and phosphorylase. Both phosphorylase and phosphorylase kinase are phosphorylated, when in the active form, which causes the breakdown of glycogen to glucose-6-phosphate which can then be metabolised further by the cell. A formidable amount of research has been undertaken and reported in an attempt to elucidate the finer points of this and other related processes and to determine the general applicability of the phenomenon. The findings in animal research considerably overshadow the equivalent research into plant tissue protein phosphorylation, so much so, that a fairly recent botanical review (Trewavas 1976a) relied almost entirely upon animal related research findings.

Numerous reviews of this aspect of animal cellular biochemistry and physiology have been published, most recently by Cohen (1980 and 1982) and by Rosen and Krebs (1981). It is not my intention to enter into undue detail with regard to animal protein phosphorylation. However to ignore these findings would leave the findings relating to plant protein phosphorylation without their experimental foundations and therefore a brief review of animal protein phosphorylation is necessary.

The phosphorylation studies, utilising animal tissues, can be placed into several categories. Firstly the activity of certain enzymes can be modulated by phosphorylation, those associated with glycogen metabolism were one of the first to be studied in detail. Secondly, in an attempt to bridge the gap between the relatively simple Jacob and Monod model for gene regulation in bacteria and the greater complexity of the eukaryotic genetic material, non-histone (or acidic) nuclear proteins have been hypothesised to act in an equivalent manner to the repressor molecules of the prokaryotic system. Nuclear acidic proteins are known to be phosphorylated and

dephosphorylated and this is thought to be a clue to their control and function. Histones, another chromatin protein component, are similarly selectively phosphorylated and although they are not now thought to play a direct role in gene regulation, this covalent modification may be an important trigger in the cell cycle sequence. Finally, a considerable amount of animal tissue and biochemical research has been directed towards an understanding of the effect secondary messengers, such as cAMP, have on the activity of protein kinases, enzymes which actually phosphorylate other proteins. cAMP, and to a lesser extent cGMP, is recognised as an important intracellular regulatory agent, which was, at least initially, thought to be ubiquitous in living organisms. The functions of cAMP in animals are well documented and researched e.g. McMahon (1974). In simple terms, cAMP participates in an intracellular messenger amplification process in which, for instance, an initial extracellular messenger (hormone) interacts specifically with a membrane bound hormone receptor which then elicits a transduction process, involving adenylate cyclase, thereby increasing the internal concentration of cAMP. In animals a common cellular hormone concentration of 10^{-12} to 10^{-9} M will induce an increased intracellular concentration of cAMP of 10^{-7} to 10^{-5} M. This evokes the physiological response which is frequently achieved by the activation of a protein kinase(s) which phosphorylates and modulates a key protein component in the physiological or biochemical sequence.

The elements of the animal cAMP systems are; (i) naturally occurring cyclic nucleotides (cAMP or cGMP). (ii) Adenylate cyclase, which converts the nucleotide triphosphate into a cyclic nucleotide. (In mammalian cells this enzyme is coupled to a hormone receptor whereas in bacteria the adenylate cyclase is hormone insensitive.) (iii) Cyclic nucleotide phosphodiesterase which is responsible for the catalytic breakdown of the 3',5'-cyclic nucleotides to 5'-nucleotides. (iv) Protein kinases.

(v) cAMP binding proteins, which are often associated as the regulatory sub-unit attached to the protein kinase molecules e.g. Langan (1973). Not all cAMP binding proteins in eukaryotes have this function, For example in *E. coli* cAMP binding protein has been shown to promote gene transcription directly (Rickenberg 1974).

The role, and indeed even the existence of cAMP in plants is still disputed. If it does exist in plant tissues then it would seem highly unlikely that it plays a similar role to that in animal systems, as its intracellular concentration is much too low to act as an amplification factor, although there may be a possibility that it acts in the way cAMP modulates activities in bacteria.

It is the intention of this report to relate a detailed investigation into the proteins synthesised and the covalent modifications of the proteins present in differentiating cells of cultured Jerusalem artichoke explants during xylem differentiation.

CHAPTER TWO

MATERIALS AND METHODS

1 Culture procedure

Helianthus tuberosus L. tubers were obtained locally and stored at 4°C in moist vermiculite/perlite in black polythene bags. The tubers were found to produce xylem in culture after up to 12 months storage under such conditions.

The tubers were washed and surface sterilised with 10% sodium hypochlorite for 15 minutes. This was followed by a brief washing of the tubers, once with sterile distilled water and once with 95% alcohol. The tubers were then flamed to remove the surface alcohol. Cylinders of tissue were obtained using a size 4 cork borer (approx. diameter 7 mm) and cut into discs (explants) approximately 1.5 mm thick using a sterile scalpel. Six of these explants were placed onto 10 ml of solidified culture medium contained in a 60 ml capacity sterile container.

All procedures were carried out in an air-flow cabinet under aseptic conditions. The cultures were maintained at 25 ± 1°C in an incubator in the dark.

Culture media

All culture media contained micro and macronutrients according to Gamborg et al. (1976) solidified with 1% agar (Oxoid technical grade 3) with the pH adjusted to 5.6 before autoclaving at 120°C for 20 minutes.

The non-xylogenic preculture medium also contained 1 mg l^{-1} (5.4 μM) 2-napthaleneacetic acid (βNAA), 5 mg l^{-1} (22.2 μM) 6-benzylaminopurine (BAP), 4% w/v soluble potato starch, 2% w/v sucrose and 0.3 mg l^{-1} (0.89 μM) thiamine HCl.

The xylogenic culture media also contained 3% w/v sucrose and various amounts of auxin and cytokinin. The medium routinely used contained 0.1 mg l^{-1} (0.45 μM) 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 mg l^{-1} (9.3 μM) kinetin (furfurylaminopurine).

The control culture media was as for the preculture medium but with 0.54 μM 1-napthaleneacetic acid (αNAA) replacing the βNAA.

2 Growth studies

Fresh weight and dry weight determinations of culture explants were obtained for each day of the culture period, by taking 10 explants which were weighed before and after drying at 60°C.

Cell numbers per explant were measured by placing the explants into 5% chromic acid for a few days, until the surrounding solution turned from orange to blue. (A modification of the original method of Minocha and Halperin 1974, as suggested by Watson and Halperin 1981). The explants were then dispersed into single cells or small clusters of cells

using a syringe (needle gauge 21) and the number of cells per explant were counted using a haemocytometer. Five explants were taken per day of the culture and 10 counts were made per explant.

The growth studies were carried out using tubers which had been stored for the periods indicated in the results.

3 *In Vivo* Radioactive labelling of the Proteins

Twenty to thirty artichoke explants, for each day of the preculture, xylogenic and control culture, were transferred aseptically to a pre-sterilised 100 ml conical flask with 10 ml of 0.01 M phosphate buffer (pH 6.8), (0.01 M Tris HCl was used in the case of the phosphorylation studies), containing an appropriately radioactively labelled chemical.

Protein synthesis was studied utilising L-[U-^{14}C] lysine monohydrochloride (342 mCi mmol^{-1}) at an incubation medium concentration of 50 µCi ml^{-1} (0.15 mmol l^{-1}). Protein glycosylation was studied using D-[U-^{14}C] Fructose (283 mCi mmol^{-1}) at a concentration of 50 µCi ml^{-1} (0.18 mmol l^{-1}). Protein phosphorylation was studied utilising carrier free [^{32}P] orthphosphate (supplied at an activity of 1 mCi ml^{-1}) at an incubation concentration of 25 µCi ml^{-1}.

The flask was placed on an orbital shaker, at room temperature, for 1.5 hours, after which time the radioactivity labelled solution was exchanged for an equivalent 'cold' solution and the flask shaken for a further 0.5 hours. All transfers were carried out under aseptic conditions, in a laminar flow cabinet, using presterilised equipment and containiners. On the completion of each labelling period a small sample of the incubation medium was taken and tested for contamination on nutrient agar (Oxoid 2.8% w/v).

4 Cell Fractionation and Protein Extraction

After incorporation of the radiolabelled precursor the explants were ground in buffer (0.01 M NaH_2PO_4, pH 7.0 containing 3 mM Potassium metabisulphite and 2 mM phenylmethylsulphonylfluoride - a protease inhibitor) at 4°C using a pre-cooled mortar and pestle. The material was then homogenised in a pre-cooled 'Atomix' blender (3 x 1 minute full speed runs, interspersed with 30 second periods of cooling in iced water). The final slurry was filtered through nylon netting (mesh size 52 µm) the filtrate being retained and the residue (cell walls and remaining whole cells) discarded.

An initial centrifugation of the filtrate (280 x g for 10 minutes at 4°C in an MSE 'Coolspin') removed any cell debris. The supernatant was further centrifuged (100,000 x g for 1 hour at 4°C in an MSE 'Prepspin'). After decanting this final supernatant the pellet was scraped from the bottom of the tube and stored at -18°C. This fraction contained the membrane-bound or particulate located protein. The final supernatant was dialysed against distilled water overnight at 4°C, freeze dried and stored at -18°C. This fraction contained the soluble or cytosol located protein.

Proteins from the particulate fraction were subsequently solubilised in the original buffer containing 0.2% Triton using a teflon-glass homogeniser. Proteins from the cytosol fraction were solubilised in the original buffer. Protein was quantified after the method of Lowry et al. (1951).

5 Electrophoresis

a) One Dimensional Electrophoresis

SDS-PAGE was carried out after the method of Laemmli (1970). Slab gels were cast between glass plates and consisted of a stacking gel (0.1% SDS, 2 mM EDTA, 0.125 M Tris-HCl, 4% acrylamide and 0.108% bis-acrylamide, pH 6.8) of dimensions 4 cm x 10 cm x 0.2 cm, and a separating gel (0.1% SDS, 2 mM EDTA, 0.375 M Tris-HCl, 10% acrylamide, 0.27% bis-acrylamide, pH 8.9) with dimensions 10 cm x 10 cm x 0.2 cm. The gel was polymerised in stages on the addition of TEMED (0.8 µl per ml of separating gel buffer and 3 µl per ml of stacking gel buffer) and ammonium persulphate (approximately 0.3 mg per ml buffer).

Protein samples were dissolved in dissociating buffer at a concentration of 1 mg ml^{-1} in glass test tubes and placed in boiling water for 3-4 minutes. (Dissociation buffer consisted of 1.25% SDS, 1% 2-mercaptoethanol, 2 mM EDTA, 10% sucrose, 0.125 M Tris-HCl, pH 6.8, and bromophenol blue to act as a tracker dye). 100 µg of protein from one of the fractions was applied to each gel track and electrophoresis was carried out at a constant current of 40 mA, at room temperature, with a running buffer consisting of 49.5 mM Tris base, 0.38 M glycine and 0.1% SDS, pH 8.3. Electrophoresis continued until the dye front reached within 1 cm of the end of the gel. Molecular weight markers were also applied to the gel and consisted of: lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), phosphorylase b (92,500), β-galactosidase (116,250) and myosin (200,000).

b) Two Dimensional Acrylamide Gel Electrophoresis

The labelled, extracted and fractionated proteins of artichoke explants were separated in two dimensions after the method of O'Farrell (1975) with modifications for the separation of plant proteins suggested by Gilbert and Buetow (1981) and Basha (1979). A two dimensional analysis of proteins involves the separation of proteins utilising two different parameters in each dimension, usually the isoelectric point of the protein in the first dimension (isoelectric focusing) and molecular weight in the second (SDS-PAGE).

i) Protein Solubilisation

The radiolabelled proteins were solubilised in the solubilising buffer (2% SDS, 0.375 M Tris base, 1 mM EDTA and 10 μg ml^{-1} butylated hydroxytoluene (BHT), pH 8.9) by placing in boiling water for 2 minutes. On cooling the solution was diluted 2:1 with the dilution buffer (5.7 g urea, 8 ml Triton X, 0.5 ml mercaptoethanol and 0.5 ml ampholytes (pH range 3-10) which was made up and stored frozen in aliquots) to give a final protein concentration of 4 mg ml^{-1}.

ii) Isoelectric Focusing Gel Preparation

The isoelectric forcusing (IEF) gels were cast in glass tubes (125 mm x 4mm external x 2 mm internal) to give a 100 mm length gel. The components of the IEF gel was as follows: 8.25 g urea, 3 ml 20% stock acrylamide, 3 ml 10% Triton X, 1.95 ml distilled water, 0.75 ml ampholytes (pH range 3-10). This gave a volume of 15 ml, sufficient for more than 15 gels. Polymerisation was induced by the addition of 10 μl of TEMED and 50 μl 10% ammonium persulphate. The IEF gel buffer was overlayed with 8 M urea prior

to polymerisation. After polymerisation the gel surfaces were rinsed with distilled water.

iii) Isoelectric Focusing

A drop of bromophenol blue was added to each protein solution to act as a marker dye. 50 µl (200 µg) of the protein solution was added to the top of the gel, which was then overlayed with 50 µl of 4 M urea and filled up to the top with the upper reservoir buffer. The gels were placed into a vertical rod electrophoretic apparatus with an acidic (anode) lower reservoir (0.06 N H_2SO_4) and a basic (cathode) upper reservoir (0.04 N NaOH and 0.02 N $Ca(OH)_2$). IEF was carried out overnight at a constant voltage of 300 V followed by a final resolution for 1-2 hours at 400 V. By this stage the bromophenol blue marker had migrated to the acidic end of the IEF gel forming a yellow band.

iv) Post-IEF

After IEF the gels were gently removed from the glass tubes with a syringe and placed into an SDS equilibrium buffer (2% SDS, 0.375 M Tris-HCl, 1 mM EDTA and 10 µg ml^{-1} BHT, pH 6.8) contained in a 100 ml conical flask on a rotating deck for 2 hours to remove the urea from the gels. The gels were then either used immediately or frozen and stored for up to 2 days.

Two gels were selected, after IEF and prior to SDS equilibration, to determine the pH gradient within the gel. The gels were sliced into 1 cm lengths and then placed in 1 ml of 50 mM KCl solution for 4 hours after which time the pH of the solution was determined using a pH meter.

v) Second Dimension (SDS-PAGE)

A 10% acrylamide slab gel was prepared, as previously described, with a 3.5 cm stacking gel. The IEF gels were placed, after SDS equilibration, over the stacking gel and held in position by pouring molten agarose (ultrapure) (1% w/v in equilibration buffer) around the gel and allowing it to set. A drop of bromophenol blue was added to the agarose to act as a marker dye and SDS-PAGE was carried out as previously described, at a constant 40 mamp. The gels were then stained, destained, prepared for fluorography and dried as previously described.

c) Gel Staining and Radiography

On the completion of the electrophoretic run the gels were stained for protein with Coomassie Blue R (0.25% w/v) in methanol: glacial acetic acid: water (45:9:46) overnight. Destaining was achieved by adding the above solvent mixture without the dye, together with approximately 1 gm of a cationic exchange resin (Amberlite RX) and incubating at about 55°C. The gels were destained in 3-4 hours.

Fluorography was used to enhance the ^{14}C- labelled precursor incorporation in the SDS-PAGE separated polypeptides (Chamberlain, 1979). After destaining, the slab gels were placed in 1 M sodium salicylate for 30 minutes then dried in a Pharmacia drier, following the manufacturer's instructions. The dried gels were clipped opposed to a Kodak 'No-screen' film (13 x 18 cm) and left for up to a month at -20°C. Autoradiography was used for the ^{32}P-phosphate labelled polypeptides, in which the above procedure was repeated but with the omission of the sodium salicylate step and exposure of the photographic plate was for 2 weeks. All films were developed using Kodak D 19 and fixed using Ilford Hypam fixative.

6 Protein Kinase Assay

The artichoke explants were homogenised as previously described but utilising a different buffer (20 mM Tris-HCl, 4 mM EDTA, and 3 mM sodium metabisulphite, pH 8.0). Explants grown on the preculture medium for 3 days were used for this set of experiments and the assay is based on that given by Keates (1973).

The kinase assay consisted of 20 µl of assay buffer (0.05 M Tris-MASA, 10 mM NaF, 0.3 mM EGTA, pH 8.0), 40 µl of casein solution (made up in the assay buffer at a concentration of 6 mg ml^{-1}). 20 µl of cytosol or particulate fraction protein (made up, after dialysis, in the assay buffer at a concentration of 10 mg ml^{-1}). 20 µl ATP-$\gamma^{32}P$ (assay concentration 32 µmoles l^{-1}, also containing 50 mM magnesium acetate) was added to begin the reaction. Radioactively labelled ATP was supplied as adenosine 5[γ-^{32}P] tri-phosphate, sodium salt (2.5 Ci $mmol^{-1}$).

The reaction was stopped by the addition of 20 µl of 0.1 M EDTA containing 50 mg ml^{-1} bovine serum albumin and transferred to ice followed by an addition of 3 ml of 10% TCA with 20 mM phosphate. The assay tubes were left at 4°C for 2 hours then at room temperature for an extra 1 hour, and collected, using a Millipore manifold, on Whatman microfibre glass-fibre filter papers (25 mm). Each filter was washed with 5 x 2 ml of the 10% TCA/phosphate then placed in a scintillation vial and dried overnight in an oven. 8 ml of scintillation fluid, 'Cocktail T', was added and the scintillation quantified using a Beckman 250. All protein kinase assays were carried out in triplicate.

7 *In vitro* Hormone Binding Assays

2,4-dichlorophenoxy (2-^{14}C) acetic acid (38 mCi mmol^{-1}) was used as the radioactivity labelled auxin. The assay method was modified from that described by Hertel et al. (1972). Two assays were carried out for each fraction. All components of the assays were dissolved in the extraction buffer.

The assay mixture for total binding contained 0.4 ml of ^{14}C-2,4-D (assay concentration 2 x 10^{-7} M), 0.4 ml extraction buffer (pH 6.8) and 0.1 ml particulate or cytosol fraction (protein concentration 0.5 to 1 mg per assay).

The assay for non-specific, non-saturable hormone binding contained; 0.4 ml ^{14}C-2,4-D (assay concentration 2 x 10^{-7} M), 0.4 ml of non-radioactive 2,4-D (assay concentration 10^{-4} M) and 0.1 ml cytosol or particulate fraction.

Binding assays with the particulate cellular fractions were allowed to incubate at room temperature for 15 minutes then centrifuged at 100,000 g at 4°C for 30 minutes. The pellet was retained and dissolved in 8 ml of Econofluor (plus 5% Protosol), then placed in a scintillation vial and incubated at 45°C overnight. Radioactivity was determined using a Beckman 250, efficiency over 90%.

Cytosol fraction binding assays were incubated at room temperature for 15 minutes. Ammonium sulphate solution was added to give a final concentration of 70% saturation and this was left for 30 minutes at 4°C. The assay was centrifuged at 50,000 g for 30 minutes and the pellet was retained. Radioactivity was determined as already described.

Saturable specific binding was taken to be the difference between the amount of ^{14}C-2,4-D bound in the two assays. (Each pair of assays were repeated in triplicate. Specific binding of 2,4-D is given as pmol 2,4-D bound per mg protein throughout this report.)

8 Chemicals

Radiolabelled chemicals were obtained from Amersham International. Plant hormones and hormone analogues were obtained from Sigma. All other chemicals were 'Analar' or as pure as obtainable.

CHAPTER THREE

XYLOGENIC CULTURES

The induction of xylogenesis in various plant tissues has been extensively reported and more recently reviewed by Roberts (1976), Shininger (1979) and Phillips (1980). Several xylem-inducing factors have been identified and include plant hormones (auxins, cytokinins, gibberellins, abscisic acid and ethylene), various metabolites (in particular, a carbohydrate source), as well as physical factors such as light and temperature. However the mere induction of xylogenesis in plant tissues is not sufficient for the detailed biochemical analysis of differentiation unless certain fairly stringent criteria are met. The criteria can be utilised either to develop an 'idealised' xylogenic culture system or to select, from those cultures available, that which is most suitable for this purpose.

The ideal xylogenic culture would have the following properties:

(i) A simple starting plant material.

(ii) Relatively simple culture techniques and manipulations.

(iii) A high yield of synchronously differentiating xylem elements.

(iv) The ability to preclude infection.

(v) The ability to dissociate the biochemical events associated with xylogenesis from other cellular chemical changes.

The first criterion is generally met in most tissue cultures, where frequently a parenchymatous tissue is utilised, as is the second where manipulations should be kept to a minimum to decrease the possibility of tissue infection. The third criterion, while being obvious, has proved the most difficult to attain. Without a high yield of xylem elements and the differentiation being, at least, fairly synchronised, then the ability to determine the biochemical modulations associated with xylogenesis can be equated with the proverbial "needle in the haystack". A frequent problem in cultured plant tissues is that of infection, which should therefore be precluded from the xylogenic cultures as it is liable to interfere with the analyses. Xylogenesis, cellular growth and division are normally closely related in plant tissues, but the biochemical details of growth and division, while probably overlapping with, are not necessarily integral to, the differentiation process. Ideally it would be advantageous to be able to identify the biochemical events occurring during normal tissue growth and to separate them from the biochemistry of xylem differentiation. This would require a xylogenic culture, the criteria for which are listed above, and a separate culture system with similar characteristics, except that xylogenesis would be negligible or absent.

The three general responses of plant tissue to the initial culture technique are growth, xylogenesis (if applicable) and a wound response. The excision of the tissue in plant explant preparation necessarily involves the wounding of the tissue, the biochemical events of which are complex (Kahl 1978). To prevent the biochemical events of wounding overlapping with and complicating the other analyses a culture period should be allowed, for at least the initial wound response to subside. (It must be added, however, that the three tissue responses are not necessarily independent of each other in the overall xylogenic process, for example the similarity between xylem elements formed in tissue culture and the wound vessel members formed in intact plants is apparent.)

The majority of the papers published regarding xylogenic induction in tissue or cell culture are of interest in their own right and do not purport to be an ideal system for the study of xylem differentiation. Many meet with certain of the criteria, but the level of xylogenesis is often less than 5% of the total cell number. A sample of such papers include work by Comer (1978) who observed 5% of the total cells differentiating as xylem in cultured *Coleus* explants, while cultured lettuce pith explants underwent 6.9% xylem differentiation (Dallesandro 1973). Mizuno et al. (1971), using carrot explants, noted between 7 and 16% of the cells undergoing differentiation into xylem, but this was over a 7 day culture period.

Jerusalem artichoke tuber tissue has regularly proved to be a useful source of material for the study of xylogenesis, exhibiting an enhanced level of xylogenesis when compared to other tissues. Dallesandro (1973) observed up to 20% xylem differentiation over an 8 day culture period, while Phillips and Dodds (1977) found approximately 15% of the artichoke cells differentiating as xylem within 2 days of a 4 day culture period.

Three culture systems maybe considered suitable for the detailed study of xylogenesis, in that a large number of xylem elements are formed under controlled experimental conditions. They are a pea root cortical explant system (Phillips and Torrey 1973), an artichoke tuber explant system (Minocha and Halperin 1974) and *Zinnia* mesophyll cell culture (Fukuda and Komamine 1980).

The maximum rate of xylogenesis in the pea root cortical explant system is 20% of the total cell number within the last 4 days of a 9 day period. The rate then decreases so that by the 24th day of xylogenic culture there are 35% of the cells as xylem. The technique for explant culture is relatively simple, although in this system it does involve the removal of the root stele from the explant. The problems of the wound response and

infection are not pertinent in the pea root system as xylogenesis begins on the 5th day of culture, sufficient time for the initial wound response to have declined and for infection of the tissues to be noted. A control explant system is utilised by the authors (and in subsequent papers e.g. Simpson and Torrey (1977) but the explants simply do not grow on the control medium, which renders it inadequate as a suitable control.

Minocha and Halperin's artichoke explant culture system is simple in terms of the starting material and the culture preparation, and the yield of xylem high, claimed to be greater than 30%. (However this estimate is probably an exaggeration due to the harsh maceration technique utilised, as discussed by Watson and Halperin (1981)). The xylogenic period is long, being the last 6 days of a 14 day culture period. This system has therefore been criticised as to its suitability for the detailed study of xylogenesis (Phillips 1980). The system also suffers from being a liquid culture in that infection will rapidly spread throughout the culture whereas a solidified culture will tend to localise infected tissues.

By far the best culture system reported to date, for the analysis of xylem differentiation, is the *Zinnia elegans* mesophyll cell culture described in detail by Fukuda and Komamine (1980) but originally noted by Kohlenbach and Schmidt (1975). In this system the cells are liquid cultured and produce a high, synchronous yield of xylem elements, up to 30% of the total cell number between days 2 and 3 of the culture period. A further advantage of the *Zinnia* system is the starting material, the first leaves of one week old *Zinnia* seedlings, which allows for the steady supply of similarly aged tissue, whereas in, say artichoke culture systems, the tubers are frequently stored. It has been noted that the response of stored plant tissue alters on prolonged storage therefore experiments intended to be comparable must utilise tissue of the same age or storage

period. There are several disadvantages in the *Zinnia* culture, for example, the starting material is highly differentiated and the practical details are complicated involving several manoeuvres, including homogenisation of the tissue, filtration and centrifugations. This, together with the low concentration of the sterilising agent (0.5% hypochlorite solution), used to avoid damaging the relatively fragile leaf tissues, makes the system susceptible to infection.

With xylogenesis occurring so early in the culture period of the *Zinnia* system it may not be possible to separate the wound biochemistry from the xylogenic biochemistry. A suitable control would suffice and while the authors demonstrated that manipulation of the culture hormone levels did indeed reduce xylem differentiation, it was not possible to determine, from the data given, if this also effected the growth of the *Zinnia* cells.

In summary, several xylogenic culture systems are available, each with certain advantages and disadvantages, with the choice of culture depending on the type of experiment to be undertaken. The ideal system for the detailed biochemical study of xylogenesis should produce a high yield of synchronously differentiating xylem elements, allow for the detection of infected tissues and be able to separate the wound, growth and xylogenic responses of the cultured tissues.

RESULTS

The original liquid culture explant system of Minocha and Halperin (1974), was repeated to give a high yield of xylem element formation. However, large scale infection was a constant problem using the liquid culture, the actual rate of xylem formation was relatively low and the high yield was indeed an artifact of the harsh maceration technique used

(Watson and Halperin 1981). A repeat of the experiment utilising the gentler maceration technique (Chapter 2, Section 2), gave a much reduced estimation of the proportion of xylem elements formed in the cultured artichoke tissues.

While there are difficulties with the original method of Minocha and Halperin, it is known that artichoke tissue does demonstrate an enhanced level of xylogenesis and therefore it was decided to develop a new culture regime to both overcome the culture problems and to attempt to increase the level of xylem formation.

i) Explant Preculture

Whereas the original culture method utilised a liquid culture, it was decided that a "solidified culture" would be beneficial in the present studies. This has the advantage of reducing cross contamination from one explant to another and making the method simpler by using stationary sterilised containers rather than liquid containing flasks on an orbital shaker.

The requirement for a preculture period, prior to xylogenesis, is of necessity for two reasons; firstly it allows a period of time to establish if the cultured tissues are free from infection, and secondly, it allows for the tissues to overcome at least the initial wound response of the excision. A variety of media were tried to determine that most suitable, consisting of a basal medium supplemented with different concentrations and classes of plant hormones. Only two of the media prevented xylogenesis during the preculture period, an obvious property desired of such a culture, one being a simple basal medium while the other was the original xylogenic medium of Minocha and Halperin, with the exception that αNAA was replaced by the same concentration of βNAA - a weak anti-auxin.

Table 3.1 demonstrates the effect the two preculture media had on subsequent xylogenesis, when the explants were placed on a xylem inducing medium. The medium containing βNAA is the preferable of the two and was used in future experiments.

Artichoke explants cultured on the preculture medium remained white when infection free but turned brown when infected. A preculture period of 3 days was determined to be a sufficient length of time to decide if an explant was infected. Uninfected explants transferred to another medium remained uninfected for the culture period with almost 100% efficiency. (Infection was determined visually and also by washing the explants with pre-sterilised phosphate buffer which was plated on nutrient agar. No bacterial colonies developed from the washings of the selected uninfected explants.)

Table 3.1 The effect of the preculture medium on subsequent xylogenesis in Jerusalem artichoke explants.

Preculture Medium	Proportion of Xylem [(d)] after 14 days on a xylogenic medium.[(b)]
No Hormones [(a)]	2.5 ± 0.8
βNAA, BAP [(c)]	14.8 ± 2.7

(a) Basal medium (B_5 and 3% sucrose) solidified with 1% agar.

(b) Original Minocha and Halperin medium, as in (c) but with αNAA replacing βNAA, and solidified with 1% agar.

(c) Basal medium plus 4% soluble potato starch and 1 mg l^{-1} (5.4 μM) βNAA and 5 mg l^{-1} (22.2 μM) Benzylaminopurine, solidified with 1% agar.

(d) A percentage of the total cell number. Mean ± SD of 10 explants.

Explants cultured on the preculture medium did not grow (Figures 3.1 and 3:2) with the number of cells per explant and the dry weight of the explants remaining the same during this period. The explant fresh weight did increase however, but this was likely to be due to water imbibition by the tissues, which had been stored for 6 months. When explants were retained on the preculture medium for extended and prolonged periods of time a small amount of growth was observed but xylogenic activity remained absent. While the explants remained viable for up to 3 months continuous culture on this medium, prolonged retention of the explants on the preculture medium did affect the level of xylem formation on subsequent transfer to the xylogenic medium (Table 3.2).

The use of a preculture period has proved very useful for the preclusion of infection and the separation of the initial wound response of the tissue from the xylogenic response, and it has also been shown to have a direct effect on the proportion of xylem formed by the artichoke explants when transferred onto the xylogenic medium (Table 3.3). As can be seen from this table, the initiation of xylogenesis is not effected by the preculture phase but the level of xylem formation is increased, over two fold, by the initial preculturing of the explants prior to their transfer onto the xylogenic culture medium.

ii) Explant Xylogenic Culture

Various concentrations of auxin and cytokinin in the xylogenic medium were tested (Table 3.4). Note that the level of xylogenesis is relatively low and that while xylem differentiation can occur in the absence of BAP it does not occur in the absence of αNAA. (The artichoke tissues possibly contain endogenous cytokinins or possess the ability to synthesise.) Of interest also is the fact that retention of the explants on the preculture

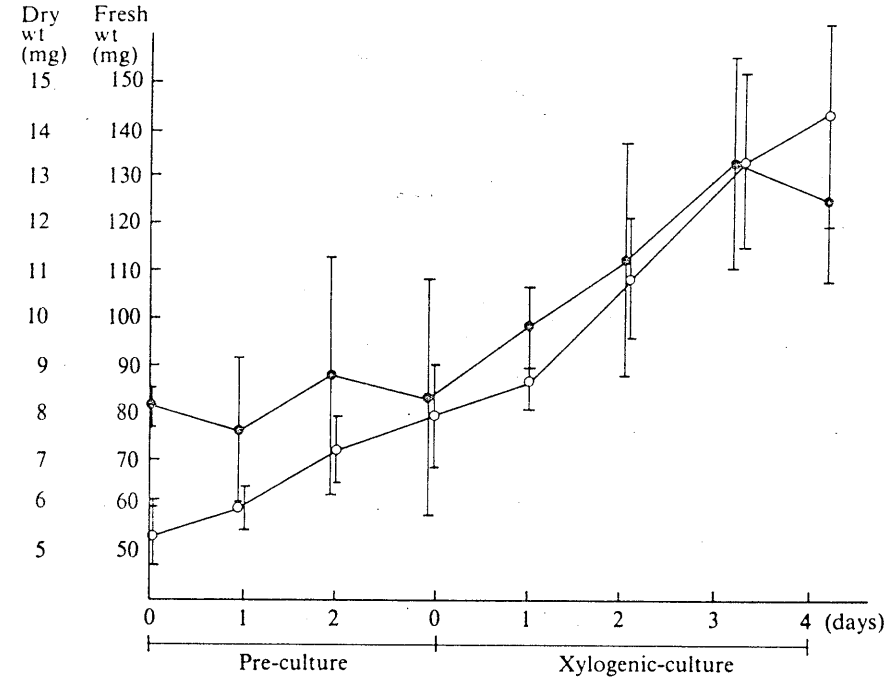

Figure 3.1 Fresh weight (open circles) and dry weight (closed circles) measurements during the culture period. (Mean ± standard deviation of 10 explants). Explants of *Helianthus tuberosus*.

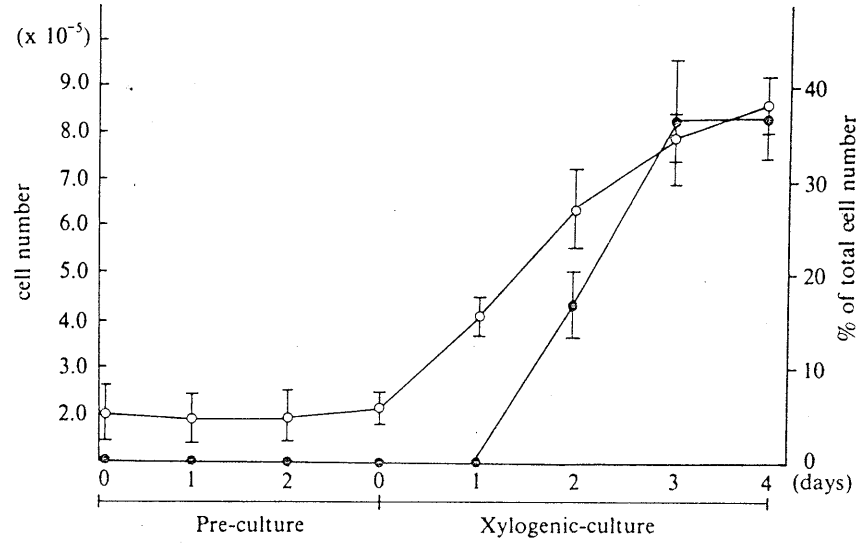

Figure 3.2 Cell number per explant (open circles) and percentage xylem per explant (closed circles). (Mean ± standard deviation). Explants of *Helianthus tuberosis*. Tubers stored for over 6 months.

Table 3.2 The effect of retention of the artichoke explants on the preculture medium on subsequent xylogenesis.

Preculture[a] Period	3 days	1 week	2 weeks	1 month	2 months	3 months	4 months
% of Xylem after 4 days (b)(c)	26.1 ± 3.1	21.5 ± 4.5	18.2 ± 2.1	18.9 ± 5.6	16.0 ± 4.1	6.3 ± 1.6	0

(a) Basal medium plus starch, βNAA, BAP and agar as in Table 3.1.

(b) Basal medium plus 0.1 mg l^{-1} (0.45 μM) 2,4-D and 2 mg l^{-1} (9.3 μM) Kinetin solidified with 1% agar.

(c) Artichoke tubers were freshly harvested. % of total cell number. Results are the mean ± SD of 10 explants.

Table 3.3 The effect of preculturing[a] the artichoke explants on subsequent xylogenesis on the xylogenic medium.[b]

Culture Period (days)	0	1	2	3	4	5	6	7	8
	← xylogenic medium →								
% Xylem[c] without Preculture	0	0	0	0	2.5 ± 1.7	7.8 ± 3.1	13.8 ± 7.6	13.3 ± 7.6	12.1 ± 3.0
% Xylem with Preculture	0	0	0	0	0.4 ± 0.1	14.8 ± 2.6	29.6 ± 5.3	31.4 ± 4.8	-
	← preculture medium →				← xylogenic medium →				

(a) and (b) as in Table 3.2.

(c) % of total cell number. Results the mean ± SD of 5 explants.

Table 3.4 The effect of different concentrations of NAA and BAP on Xylogenesis in cultured artichoke explants.[a]

	0 mg l^{-1} αNAA	0.1 mg l^{-1} αNAA	1 mg l^{-1} αNAA	2 mg l^{-1} αNAA	1 mg l^{-1} βNAA
0 mg l^{-1} BAP	0[a]	-	5.0 ± 1.1	-	-
5 mg l^{-1} BAP	0	4.3 ± 1.2	12.1 ± 3.4	8.5 ± 3.1	0

(a) The percentage of total cell number as xylem formed after 3 days preculture and 7 days xylogenic culture. The results are the mean ± SD of 5 explants.

(b) The xylogenic medium consisted of a basal medium plus 4% starch and the exogenous plant hormones indicated in the table. The medium was solidified with 1% agar.

medium (1 mg l^{-1} βNAA, 5 mg l^{-1} BAP) prevents xylogenesis.

The level of xylem differentiation was considered to be insufficient for the detailed analysis of the associated biochemistry, therefore 2,4-D and kinetin were used, in a similar experiment to that in Table 4, to replace αNAA and BAP (Table 3.5). The peak level of xylogenesis occurred with the hormone combination of 0.1 mg l^{-1} 2,4-D and 2 mg l^{-1} Kinetin BAP, this was used in all future experiments as the 'xylogenic medium'. It is interesting to note that, at the maximal auxin level (0.1 mg l^{-1} 2,4-D) the effect of altering the cytokinin concentration was not as marked as altering the auxin concentration at the maximal cytokinin level (2 mg l^{-1} kinetin) on the number of xylem formed. Auxin would appear to be the more important exogenous plant hormone addition for the induction of xylogenesis, while cytokinin probably increases the number of xylem elements being formed by simply increasing the number of cells available for differentiation.

Figures 3.1 and 3.2 demonstrate that the fresh weight, dry weight and cell number per explant increase on transfer of the explant onto the xylogenic medium, but declines by day 4 of this culture period. The proportion of xylem elements per explant (as a % of the total cell number), increases rapidly between days 1 and 3 of the xylogenic culture period (Figure 3.2), with the maximum rate of xylogenesis occurring on days 2 and/or 3, varying slightly from culture to culture.

Experiments using artichoke tubers can be continued throughout much of the year, between 'natural' harvests, by storing the tubers (as outlined in the methods section). The tubers remain visibly dormant from the initial storage, in November, until April or May, when roots and etiolated shoots form on the tubers, which indicates a probable alteration in the biochemical balance of the artichoke tissue. The biochemical responses of the tissue will, therefore, depend upon the length of time

Table 3.5 The effect of different concentrations of 2,4-D and kinetin on Xylogenesis in artichoke explants.[a]

	0 mg l^{-1} kinetin	0.1 mg l^{-1} kinetin	0.4 mg l^{-1} kinetin	1 mg l^{-1} kinetin	2 mg l^{-1} kinetin	3 mg l^{-1} kinetin	4 mg l^{-1} kinetin
0 mg l^{-1} 2,4-D	0[b]						
0.001 mg l^{-1} 2,4-D					0		0
0.01 mg l^{-1} 2,4-D		3.4 ± 1.7		4.7 ± 1.5	4.2 ± 2.3		
0.05 mg l^{-1} 2,4-D					5.1 ± 1.7		
0.1 mg l^{-1} 2,4-D			14.5 ± 3.5	15.3 ± 4.4	30.6 ± 6.1	21.8 ± 6.3	24.6 ± 2.9
0.2 mg l^{-1} 2,4-D					21.9 ± 7.9		

(a) The culture medium consisted of the basal medium and the exogenous plant hormones indicated in the table. The medium was solidified with 1% agar. The explants were precultured prior to their culture on the xylogenic medium for 4 days.

(b) The % of xylem elements as a proportion of the total cell number. Each result is the mean ± SD of 5 explants. The artichoke tubers had been stored for 5 months.

the tubers have been stored. At all stages of this report, experiments which have been intended to be comparable have been carried out within as short a time as possible, never more than one week between two comparable experiments. Several changes have been noted in the tuber tissues throughout the storage period, one such change being the actual level of xylogenesis in the explants, (Table 3.6), which increases on storage of the tissue.

iii) Explant Control Culture

Ideally the control artichoke culture should allow the explants to grow at the same rate as the explants cultured on the xylogenic medium, but should not allow the differentiation of xylem elements. The original work of Minocha and Halperin noted that at the hormone concentrations of 0.1 mg l^{-1} αNAA and 5 mg l^{-1} BAP the proportion of xylem elements produced by the explants was much reduced while cellular growth remained comparable to the explants undergoing large scale xylogenesis. I repeated this finding (Table 3.4) determining that, at these concentrations of hormones, only approximately 4% of the cells differentiated as xylem elements, and that, on the 4th day of the culture period. (This was not simply a delayed production of xylem, as explants cultured on the control medium for longer periods did not increase the proportion of xylem formed.) A comparison of the dry weight and fresh weight increases in explants grown on the control and the xylogenic media, Figures 3.3 and 3.4, show that they are very similar. These findings comply with the requisites for a 'control culture' and as such was therefore utilised in future experiments.

Table 3.6 The effect of artichoke tuber storage on the level of Xylogenesis in cultured artichoke explants.[b]

Storage Period (months)	% Xylem[a]
Freshly Harvested	23.7 ± 2.1
2	27.6 ± 4.1
4	30.5 ± 5.6
6	28.2 ± 3.6
9	36.0 ± 5.8

(a) % of total cell number. The results are the mean ± SD of 5 explants.

(b) Explants were precultured prior to being placed on the xylogenic medium for 4 days.

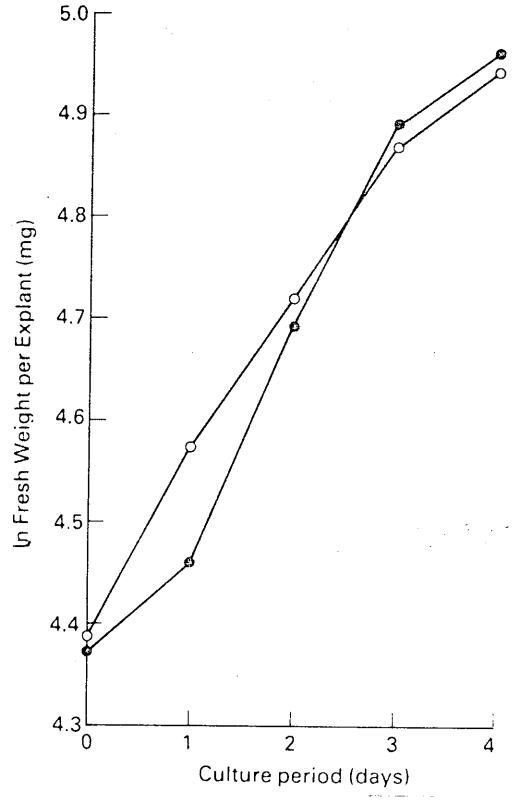

Figure 3.3 Fresh weight measurements of artichoke explants cultured on the xylogenic medium (closed circles) and the control (non-xylogenic) medium (open circles). Each value is the mean of 10 explant weighings.

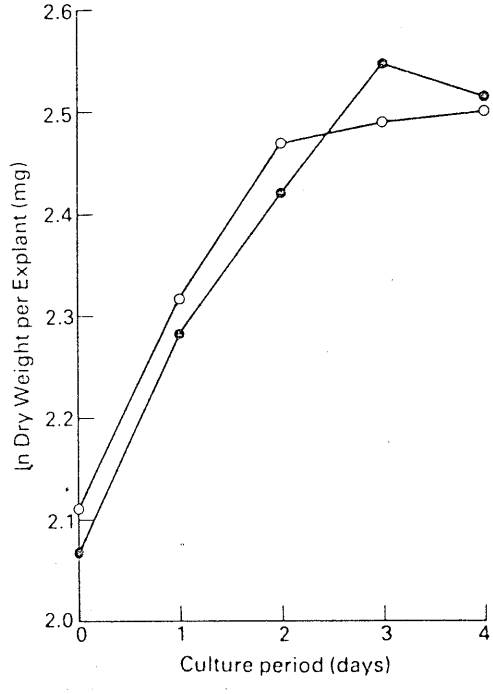

Figure 3.4 Dry weight measurements of artichoke explants cultured on the xylogenic medium (closed circles) and the control medium (open circles). Each value is the mean of 10 explant weighings.

DISCUSSION

The artichoke explant culture regime described in this section fulfils the criteria set out for a suitable system for the detailed biochemical study of xylogenesis. It is a two step technique in which tissue infection can virtually be eliminated prior to xylogenesis. A high yield of xylem elements, up to 36% of the total cell number, depending on the storage age of the tubers, can be obtained using this system. Even the lower yields, observed when using freshly harvested tubers, are still sufficient to comply with the criteria outlined. The short period in which xylogenesis occurs, within 2 days of a 4 day culture period, is ideal for biochemical studies, as is the control culture, which allows the explants to grow at an equivalent rate to that of the explants undergoing large scale xylogenesis but without a significant production of xylem elements.

The preculturing of the explants is an important means of selecting uninfected explants for further growth, differentiation and analysis. Interestingly, the preculture act in itself appears to have an enhancing effect on the subsequent xylogenic activity of the explants. The weak anti-auxin, βNAA, appears to prevent cell division and growth during the preculture period, which allows for the possibility that it causes the synchronisation of the precultured explant cells. This is indicated by the immediate growth, without a lag period, of the transferred explants on the xylogenic and control media. Explants retained on the preculture medium, while not growing over the initial period of the culture, do begin to grow slowly after several weeks retention on this medium. This is probably due to the production, by the explants, of endogenous plant hormones.

The two step artichoke explant culture regime is an improvement on the pea root cortical explant system and the original artichoke explant culture,

in that a high yield of xylem elements differentiate over a much shorter culture period and the infection of the tissues can be precluded prior to xylogenesis. It is also an improvement on the *Zinnia* mesophyll cell culture in that the artichoke tuber starting material is a simpler tissue than the leaf tissue of *Zinnia*. The method of culture preparation in the new artichoke system is far less complex than those detailed in the preparation of the pea root cortical explants and the *Zinnia* mesophyll cultures. While the rate of xylem element formation in the new artichoke system is lower than in the *Zinnia* culture, the ability to preclude infection gives the artichoke culture a significant advantage over the *Zinnia* culture for the detailed biochemical study of xylogenesis.

The two step regime should provide the possibility of separating the initial wound response of the tissue from the subsequent xylogenic development and its analysis. This is not to say that the wound response of the tissue is not an integral aspect of xylogenesis in this system, for example, the resemblance of the xylem elements formed in tissue culture to the wound vessel members formed in intact plant is apparent. Importantly, however, the plant tissue wound responses are complex reactions to the excision and tissue culture preparation technique, causing various syntheses and activations of cellular constituents which could well mask the biochemical events of xylogenesis, unless separated in time from the xylogenic response of the tissue. This is certainly a problem with the *Zinnia* culture in which xylem differentiation occurs very soon after the culture preparation during such a period at which time the wound events are probably still continuing. The 3 day preculture period should allow for, at least, the initial wound response to have subsided. Similarly, the explants grown on the xylogenic medium are expressing at least two other responses; xylem differentiation and growth. The ability to separate the events of growth from those of xylogenesis is an obvious advantage and should be possible with the use of the control culture.

A disadvantage in the use of artichoke tuber tissues, in the above studies, is the fact that the tubers are stored after harvesting. The tubers are seen to alter throughout the storage period, both morphologically and in the tissues response to additions of exogenous hormones and biochemical inhibitors. This problem can be overcome by ensuring that experiments, intended to be comparable, are executed within as close a proximity of time as possible.

In summary, the culture regime in this report goes some way to meeting the criteria of suitability for a system capable of being utilised for the detailed biochemical analysis of xylem differentiation. Artichoke explants are excised, placed on a preculture medium for a period of three days, after which time, the uninfected explants are transferred either to a xylogenic medium or a control medium. It should be possible utilising this system to analyse aspects of xylem differentiation separated from the initial wound response and normal growth of the cultured tissues.

CHAPTER FOUR

AUXIN-SPECIFIC BINDING IN CULTURED ARTICHOKE EXPLANTS

The presence of plant hormone receptors implies the hypothesis that a particular hormone controls the observed response of the tissue or cell by firstly binding to a specific receptor which then, either directly or through a secondary messenger or reaction, controls the mechanics of the hormone interaction with the system. Auxin specific binding proteins (reviewed by Rubery 1981) have been isolated from intact and cultured plant tissues, although the necessary criteria needed to designate any as a 'true' receptor still require fulfilling.

The criteria used to determine whether a hormonal binding is of physiological significance (Kende and Gardner 1976) are related to the structural and tissue specificity of the analysed binding and the measured kinetic parameters of this binding. Structural specificity should be displayed by the binding factor, in that structurally related hormones or analogues will compete for the binding site while non-related chemicals, including other classes of hormone, will not. Tissue specificity, a criterion used successfully in animal studies, is not necessarily applicable in the study of plant receptors in that the same plant hormones have a variety of regulatory roles in most if not all plant tissues, thus a narrow tissue specificity is unlikely to be observed in plants. The

The kinetic parameters of the hormone binding site should correspond to the kinetic response of the tissue it was extracted from, the kinetic parameters being determined by using a variety of assays, the basic concept of which is to have a radiolabelled ligand (the hormone or analogue) in equilibrium with the binding site. In reality the assay must also be able to distinguish between specific binding of the ligand and the often large amounts of non-specific binding of the ligand. This is achieved by correcting the total bound radioactivity, determined in the assay, by subtracting the radioactivity measured in an identical assay but in the presence of excess (10^{-4} M) non-radioactive hormone. (This should remove the specifically bound radiolabelled hormone but not the non-specific fraction.)

There are several practical methods utilised to determine specific hormonal binding in plants. For the cytosol located binding sites (or solubilised particulate binding sites) equilibrium dialysis (Venis 1977), gel filtration (Cross and Briggs 1979), dextran coated charcoal absorption of free ligand (Oostrom et al. 1980) and precipitation by ammonium sulphate (Wardrop and Polya 1980) have all been used to separate bound and free ligand. In particulate cellular fractions; filtration (Trillmich and Michalke 1979) and centrifugation (Hertel et al. 1972) have been utilised.

The kinetic parameters are usually derived from a Scatchard plot (Scatchard 1949), which is a representation of data derived using one of the above methods. The amount of bound radiolabelled ligand is plotted against the ratio of the bound to unbound ligand. The slope of the graph is the negative reciprocal of the affinity of the binding site (called the K_D value) and the abscissal intercept indicates the number of binding sites per assay (usually uniformised with the tissue fresh weight or the amount of protein in the assay). Binding heterogeneity may be deduced

from a Scatchard plot with a non-linear graph, especially if there is a sharp break in this line. The data for this plot are generated by decreasing the effective concentration of the radiolabelled ligand, in the assay, by increasing the amount of an identical, but non-radioactive, ligand. A high affinity binding site should have a K_D value similar to the effective tissue concentration of the hormone concerned. (This is often difficult to determine, as well as the fact that the hormone may well be effective over several magnitudes of concentration.) A general rule of thumb is that a binding site is thought to be of possible physiological significance if the K_D value is less than 20 μM.

Cellular components, other than 'receptors', will specifically bind plant hormones, for example, hormone transport molecules and enzymes involved in the metabolism of the hormone. The kinetic parameters of the putative 'receptor' should help to eliminate the other possibilities. It is interesting to note (Murphy 1979) that, by utilising the criteria listed above for the validation of hormone specific binding, Bovine serum albumin, if it had been extracted from plant tissues, would demonstrate auxin specific binding and could be considered as a putative receptor! Obviously great care must be exercised before designating a receptor function to any factor under analysis.

The major advances in auxin binding studies have revolved around research on dark grown maize coleoptiles. The pioneering work of Hertel et al. (1972), Lembi et al. (1971) and Thomson et al. (1974) has since been refined to reveal several distinct auxin binding sites in various intracellular locations (Batt et al. 1976, Ray 1977, Ray et al. 1977, Dohrmann et al. 1978 and Jacobs et al. 1978). Auxin binding in this system has been optimised to uncover 3 separate binding sites with distinct auxin specificities and subcellular locations as well as an

auxin binding inhibitory factor located in the supernatant and identified as a glucoside of 6,7-dimethoxybenzoxazinone (Venis 1978).

The initial studies on washed microsomes, of fractionated maize homogenate, showed a binding site for 1-NAA with a K_D of 0.5 to 0.7 μM and a tissue concentration of 50 pmol per gm fresh weight. This site was located in the endoplasmic reticulum (Ray 1977). Auxin binding site II has a lower affinity for 1-NAA and 2-NAA and its location is uncertain, although thought to be at the tonoplast. Site III preferentially binds IAA and 2,4-D rather than NAA, with a K_D of 5 μM and a concentration of 40 pmol per gm fresh weight (Dohrmann et al. 1978). Its location is thought to be the plasma membrane. Similar research by Batt and Venis (1976) has shown a major (K_D of 1.6 μM at 32 pmol per gm fresh weight) and a minor (K_D of 0.39 μM at 24 pmol per gm fresh weight) NAA binding site in the particulate fraction of maize coleoptiles. Particulate located auxin binding sites have been established in plant sources other than maize, including: *Cucurbita* (Jacobs and Hertel 1978), cultured tobacco pith explants and callus (Oostrom et al. 1975) and also in cultured explants of Jerusalem artichoke (Trewavas 1980).

Non-membranous (cytosol) located auxin binding sites have also been demonstrated and characterised. Wardrop and Polya (1977) demonstrated a soluble binding protein in bean leaves. It has a high binding capacity and a high affinity for auxin (K_D of 0.8 - 0.3 μM). This factor has been shown to be the enzyme ribulose-1,5-bisphosphate carboxylase and although it is not thought to play a direct role in signal transduction it may play a role in the sequestration of free auxin (Wardrop and Polya 1980 a and b). A soluble auxin binding protein has also been extracted from cultured tobacco pith explants (Oostrom et al. 1975 and 1980).

The large potential for control auxin specific binding proteins would possess, assuming that they have a similar role to that of animal hormone receptors, makes them a suitable point of investigation as a possible modulatory focus during cytodifferentiation. A role in the transduction of an initial step in differentiation has obvious appeal as part of a model for xylem induction, acting either directly on the genome or at the plasma membrane where a secondary response would amplify the initial interaction. Xylem differentiation in cultured artichoke explants is controlled predominantly by auxins, with 0.5 μM 2,4-D causing large scale differentiation in the tissue. It was therefore considered to be of interest to ascertain, firstly if an auxin binding factor was present in the artichoke tissue and secondly, to determine if any quantitative changes in the auxin binding factor(s) could be correlated with the xylogenic process.

RESULTS

i) Optimum Conditions for 2,4-D Binding

The tissue fractions used to determine the optimum conditions for the subsequent hormone binding experiments were pooled fractions obtained from explants cultured on the xylogenic medium from one to four days.

The time course for specific 2,4-D binding to the cellular fractions was determined by allowing the assays to incubate for increasing levels of time. The binding was very rapid, with 90% occurring within one minute. (Figure 4.1 shows the time graph for the particulate fraction.) A similar graph was obtained using the cytosol fraction. All future assays were incubated for 15 minutes after which time 99% of the binding had taken place.

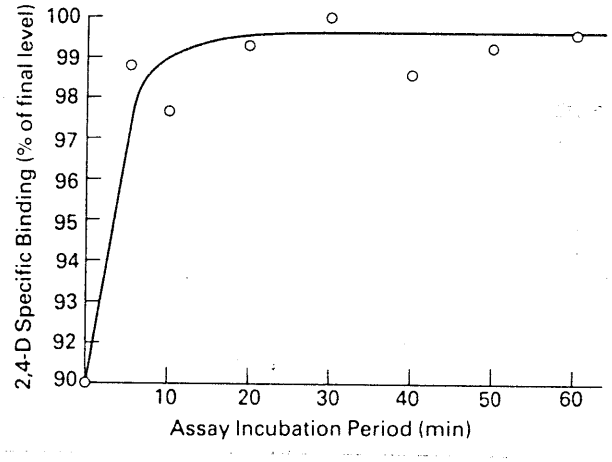

Figure 4.1 Time course of ^{14}C-2,4-D binding to pooled particulate fractions extracted from artichoke explants cultured on the xylogenic medium.

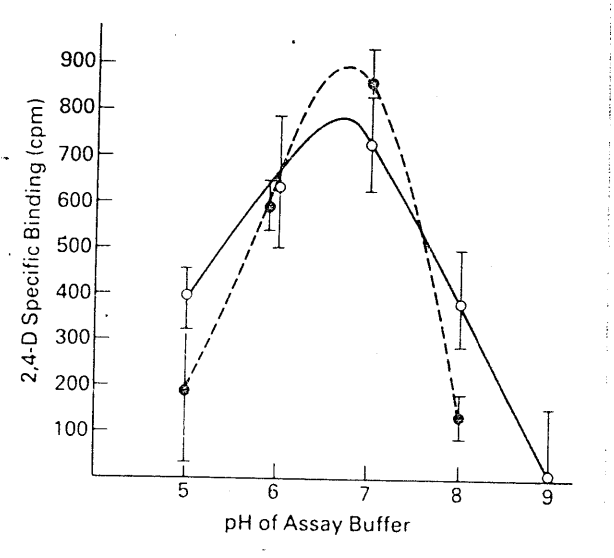

Figure 4.2 The pH dependence of ^{14}C-2,4-D binding to the particulate fractions (open circles) and the cytosol fractions (closed circles) extracted from artichoke explants and pooled over the xylogenic period. (Each value is the mean ± SD of 3 estimates).

The pH dependance of the 2,4-D binding was demonstrated by determining the amount of specifically bound ^{14}C-2,4-D in assays of various pH (Figure 4.2). Similar graphs were obtained with the cytosol and particulate fractions, the cytosol binding factor having a slightly narrower binding pH range. Future assays were carried out at the optimum pH of 6.8.

ii) The Chemical Nature of the 2,4-D Binding Factor

The binding factor was found to be heat labile. The specific binding by the factor was reduced by up to 90% when the cellular fractions were heated at 60°C for 20 minutes.

The protein nature of the binding factor was further indicated by the use of phenylmethylsulphonylfluoride (PMSF), a protease inhibitor, in the binding/extraction buffer. Cellular fractions which had been stored for 3 months frozen in the buffer minus PMSF exhibited approximately a 50% reduction in the specific binding when compared to the same fractions stored in identical conditions but with the addition of 2 mM PMSF. Binding assays carried out prior to storage showed no difference with and without PMSF.

iii) Kinetic Parameters of the 2,4-D Binding

The affinity of the specific binding sites and their concentration in cultured artichoke tissues were determined from Scatchard plots. The assays were carried out using fractions obtained from explants which had been grown on the xylogenic culture medium for 2 days. Assays were carried out as previously but with increasing amounts of non-radioactive 2,4-D added to compete for the saturable specific 2,4-D binding sites.

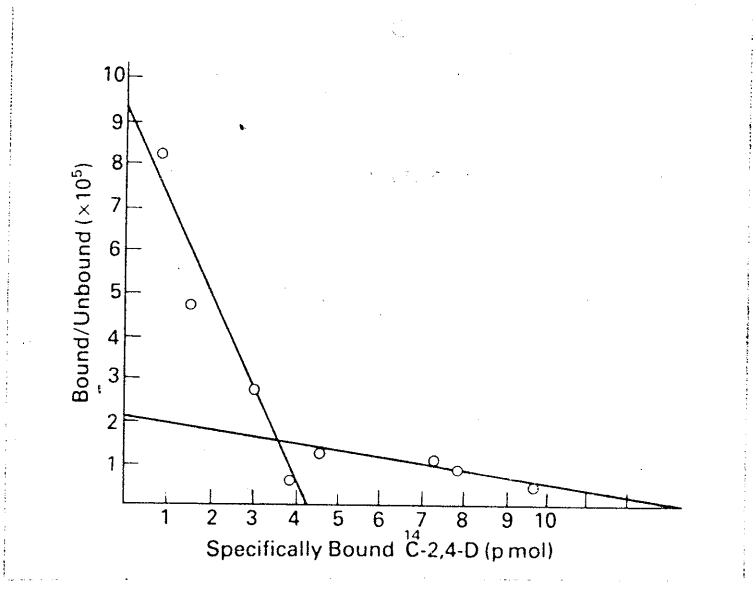

Figure 4.3 Scatchard plot of data determined using the particulate fraction extracted from artichoke explants cultured on the xylogenic medium for 2 days.

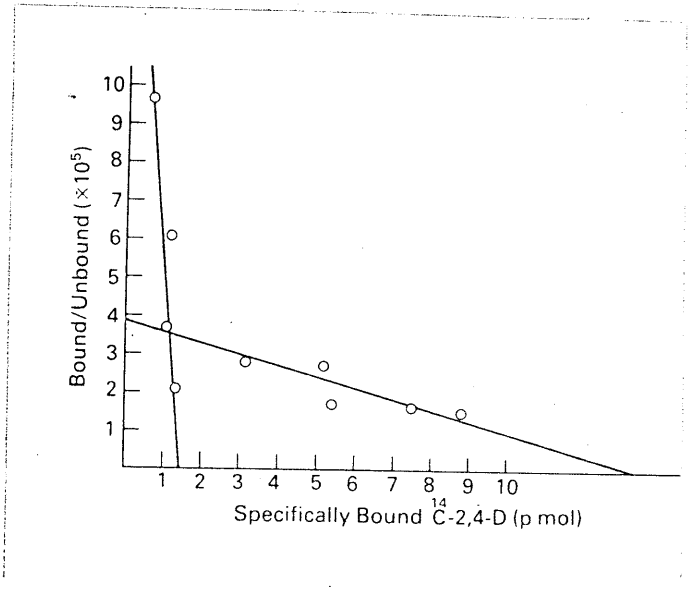

Figure 4.4 Scatchard plot of data determined using the cytosol fraction extracted from artichoke explants cultured on preculture medium for 3 days.

The particulate fraction of the artichoke explant homogenate displayed 2 binding sites: a high affinity site (K_D of 8.7×10^{-9}M) at a concentration of 0.95 pmol per mg protein (approximately 0.35 pmol per gm fresh weight) and a slightly lower affinity site (K_D of 3.2×10^{-7}M) at a concentration of 7 pmol per mg protein (approximately 2.6 pmol per gm fresh weight) (Figure 4.3).

The cytosol fraction gave a very similar Scatchard plot (Figure 4.4) again with two binding sites: K_D of 4.5×10^{-8}M, concentration of 1.1 pmol per mg protein or 0.37 pmol per gm fresh weight and a K_D of 6.3×10^{-7}M, concentration of 3.2 pmol per mg protein or 1.1 pmol per gm fresh weight.

iv) Specificity of the 2,4-D Binding Protein

Binding assays were carried out as normally but in the presence of non-radioactive plant hormones of various classes: auxins, cytokinins and a gibberelin, to determine their abilities to compete for the 2,4-D binding site.

Table 4.1 shows the effect of the various hormones on the 2,4-D binding to the cytosol fraction. Both the natural auxin, IAA, the synthetic auxin, αNAA, and a weak anti-auxin, βNAA, compete readily with the ^{14}C-2,4-D for the binding sites, however, none competed as well as the 'cold' 2,4-D. GA_3 had no significant effect on the ^{14}C-2,4-D binding while the cytokinins slightly enhanced the 2,4-D binding, but not significantly.

When the experiment was repeated using the particulate fraction, none of the plant hormones had any significant effect on the ^{14}C-2,4-D binding i.e. there was no competition for the auxin binding site.

Table 4.1 Effect of various plant hormones on 2,4-D binding to the cytosol fraction of Jerusalem artichoke homogenate. (Explants cultured for 3 days on a pre-culture medium).

Plant Hormone Competitor (a)	2,4-D Specific Binding (cpm) (b)	Specific 2,4-D Binding Uncompeted (%)
2,4-D Dichlorophenoxy-acetic acid	(297.9 ± 37.0)	(0) (c)
α Napthaleneacetic acid	151.5 ± 37.8	50.9
β Napthaleneacetic acid	146.5 ± 42.1	49.2
Indoleacetic Acid	170.7 ± 29.5	57.3
Gibberlic acids	273.4 ± 73.6	91.8
Kinetin	364.7 ± 27.8	122.4
Benzylaminopurine	303.5 ± 51.2	101.9

(a) Hormone added to assay at a concentration of 10^{-4} M in place of the 'cold' 2,4-D.
(b) Determined as described in methods section mean ± SD of three assays.
(c) Specific binding determined for 2,4-D designated as 100%.

v) Modulation of the Tissue Concentration of 2,4-D Binding Sites during culture in different media.

Artichoke explants were cultured on pre-culture medium, xylogenic medium and a control medium, with 2,4-D specific binding assays being carried out for each day of a four day culture period (3 days for the pre-culture) in order to observe changes in auxin binding site numbers within the cultured artichoke explants.

Figures 4.5, 4.6 and 4.7 show the auxin binding site concentration throughout the culture period on each of the three culture media. When artichoke explants were excised and placed on the pre-culture medium there was a rapid rise in the concentration of auxin binding sites, in both the cytosol and particulate fractions, peaking on day 1 of the pre-culture period (Figure 4.5). This is possibly associated with a wound response of the tissue.

On transfer of the pre-cultured, uninfected explants onto a xylogenic medium (Figure 4.6) there is again a peak of 2,4-D specific binding, after a lag of 1 day, in the particulate fraction. This peak of auxin binding coincided with the initial major increase in xylem formation in this system.

However when similar experiments were carried out using explants grown on the control medium (i.e. growth without xylogenesis) a very similar graph was obtained, although the peak in the particulate associated auxin binding occurred a day later (Figure 4.7).

The cytosol associated auxin binding sites were absent on the initial excision of the explants, rose in concentration after the first day of the culture and remained relatively steady except for the slight decline on transfer to a new culture medium.

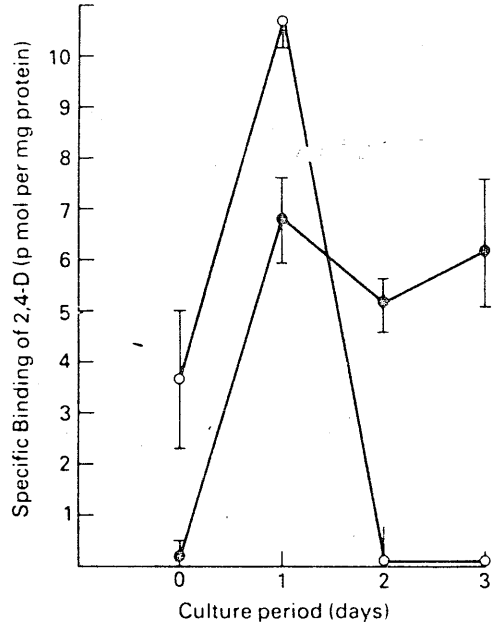

Figure 4.5 Changes in 2,4-D specific binding in the particulate fraction (open circles) and cytosol fraction (closed circles) of artichoke explants cultured on the preculture medium. Values are the mean ± SD of 3 separate experiments.

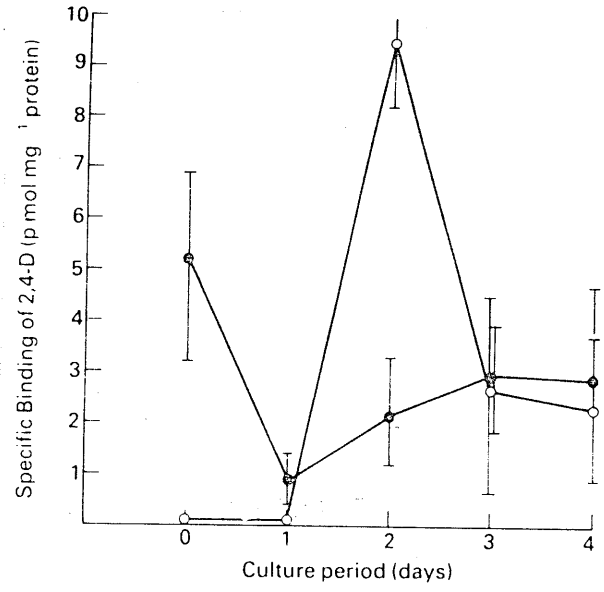

Figure 4.6 Changes in 2,4-D specific binding in the particulate fraction (open circles) and cytosol fraction (closed circles) of artichoke explants cultured on the xylogenic medium. Values are the mean ± SD of 3 separate experiments.

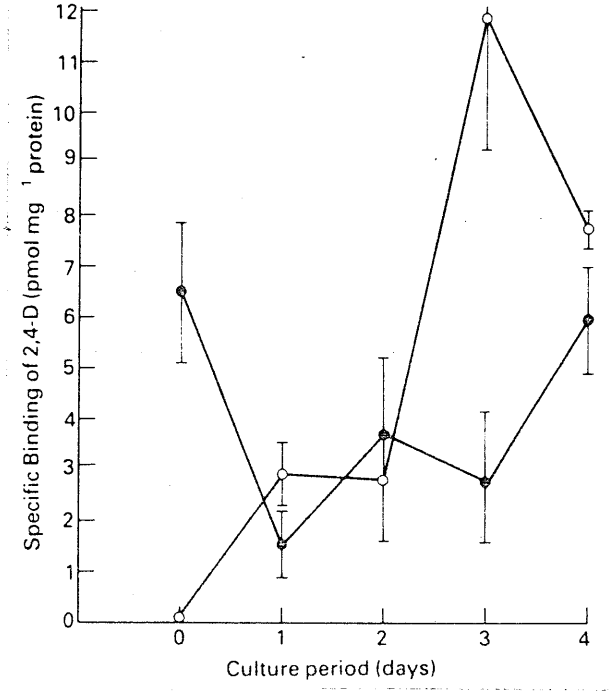

Figure 4.7 Changes in the 2,4-D specific binding in the particulate fraction (open circles) and cytosol fraction (closed circles) of artichoke explants cultured on the control (non-xylogenic) medium. Values are the mean ± SD of 3 separate experiments.

DISCUSSION

The concentration of specific binding sites for auxin has been shown to change in cultured plant tissues, for example, in tobacco pith (Oostrom et al. 1975) cytoplasmic auxin binding sites were not detectable in the original plant tissue but did appear on culturing of pith explants. The pH optimum for this IAA binding was 7.5 with K_D values ranging between 10^{-7} and 10^{-8} M and tissue concentration of between 10 and 50 x 10^{-15} mol per mg protein in the callus. On transfer of the callus to fresh medium, every 21 days, the number of IAA binding sites rose gradually to peak at culture day 12 followed by a sharp decline to very low levels by day 18 (Oostrom et al. 1980). A study of particulate located NAA binding sites in batch cultured tobacco cells (Vreugdenhil et al. 1981) gave very similar results. In this liquid culture, with a subculture period of 10 days, the concentration of auxin binding sites increased gradually up to 6 days and then declined rapidly by day 8. While modulations in the number of auxin binding sites during culture have been reported, there has been no attempt to designate a function for these fluctuations.

The 2,4-D binding protein isolated from artichoke tubers has an optimal binding at pH 6.8, is saturable (K_D's of 3.2 to 6.3 x 10^{-7} M and 0.87 to 4.5 x 10^{-8} M) and changes in tissue concentration during culture from 0 to 12 pmol per mg protein. This binding has been detected in both the cytosol and particulate fractions of the artichoke explant homogenate and, from the similarities of the optimal binding conditions and the kinetic parameters of each, there is a strong indication that the protein in each fraction is the same. However, the findings relating to the auxin specificity were strangely dissimilar, with the cytosol located binding protein demonstrating the specificity expected of an auxin specific binding factor whereas the particulate located factor, while binding 2,4-D, did not demonstrate the characteristic competition response to other auxins, although

neither fraction showed any significant binding of other classes of plant hormones. Although ammonium sulphate precipitation has been shown to give misleading results in the past (e.g. the auxin-binding protein in pea and bean leaves was determined to be ribulose-1,5-bisphosphate carboxylase (Wardrop and Polya 1980) and did not have a physiological role in the binding of auxins) there are points in favour of the cytosol located binding protein being at least a specific 2,4-D binding factor and not just an artifact of the assay technique. The cytosol located binding protein has demonstrated that its 2,4-D binding can be competed by other auxins but not by other classes of plant hormones, the fluctuations in tissue concentration during the various explant cultures varied reproducibly throughout the culture period and the binding parameters of the cytosol located factor are very similar to that of the particulate located factor (which was measured using a different and acceptable procedure). With regard to the lack of competition witnessed in the particulate fraction (Vreugdenhil 1980), studying particulate located NAA binding sites in tobacco leaf protoplasts, concluded that during protoplast preparation the auxin binding sites were damaged or destroyed. Something of a similar nature could be happening to the particulate located binding sites during the artichoke fractionation procedure. However, Dollstadt et al. (1976), investigating the specific binding of IAA and phenoxyacetic acid derivatives (e.g. 2-methyl-4-chlorophenoxyacetic acid; MCPA) in membranous fractions of pea observed specific binding for both the above chemicals with a K_D of $\leq 10^{-6}$ M and a tissue concentration of ≤ 0.1 pmol per mg protein. However in displacement experiments the phenoxyacetic acid derivatives were able to displace IAA but IAA was not able to displace the derivatives. The authors concluded that the herbicides (phenoxyacetic acid derivatives) bound to a different site than that of IAA, but one which was allosterically connected to it. Certainly this result would be in agreement with my findings for the 2,4-D binding in the particulate fraction of artichoke but does not explain the difference between the cytosol located and the

particulate located binding protein, which were so similar in many other factors.

The artichoke 2,4-D specific binding noted in this investigation is very similar to that found in the particulate fraction of artichoke tuber tissues previously (Trewavas 1980). He assayed auxin binding using ^{14}C-IAA in explants cultured in a medium supplemented with 2,4-D, which was demonstrated to induce IAA binding factors, or on a control medium without 2,4-D, which failed to induce IAA binding factors. The *in vitro* binding was carried out at a pH of 5.5 (no binding took place above pH 7.4) with a K_D of c.a. 10^{-6} M. The fluctuation of binding site concentration in the membranous fraction of the cultured artichoke is very similar to that of the changes noted in the pre-culture period in this investigation and a very limited displacement experiment demonstrated that the IAA binding could not be displaced by gibberellic acid or abscisic acid (although other auxins were not apparently tested). While minor differences are apparent between the binding site reported by Trewavas and that in this report, several of the differences could be accounted for by the different pH of the assay buffer, it would therefore appear that the binding sites are very similar. (Trewavas did not undertake an investigation into the cytosol fraction of the artichoke homogenate.)

Bogers et al. (1980) suggests that the initial increase in the concentration of auxin binding sites in tobacco pith explants, within one or two days of tissue excision, is likely to be associated with a wound response of the tissue. Many biochemical changes do occur on the excision or 'wounding' of plant tissues (Kahl 1978), but how the changes in auxin binding site concentration relate to these biochemical responses require further investigation. While Trewavas (1980) concluded, with regard to the IAA binding site located in the membranous fraction of artichoke, that the

increase in the number of binding sites on culture period, were either a real increase in number induced by 2,4-D, or an apparent increase caused by the protection of the IAA binding sites by 2,4-D which are damaged on extraction from the tuber tissue in the absence of another auxin or analogue. βNAA, used in this investigation, could have a similar effect.

As well as the initial peak of binding site number on excision of the tissue, a further peak of 2,4-D binding site concentration occurs on transfer of the pre-cultured artichoke explants onto another culture medium. The pattern of binding site fluctuations were very similar in explants undergoing large scale differentiation (xylogenesis) and also explants simply growing. The change in binding protein concentration, therefore, had no correlation with the cytodifferentiation process and is probably a 'transfer' or 'subculture' response of the tissue, as noted in the tobacco cultures. Again the role of the changes observed cannot be designated a function but it does not appear to be related to the growth of the tissue. Firstly the peak witnessed in the pre-culture period occurs in the absence of tissue growth and secondly, on transfer of the pre-cultured explants to either the xylogenic or control medium, there was immediate growth of the cultured tissues, whereas the increase in the concentration of 2,4-D binding sites lagged behind this.

The possible receptor functions of auxin binding proteins can be collated into 3 categories: the control of ion pumps (and growth), the control of auxin transport and the control of gene expression. An attempt to correlate primary auxin binding with the extrusion of protons and cell enlargement (the acid growth hypothesis) has been attempted, but as yet without conclusive evidence in its favour. Auxin transport is probably the most likely function, in terms of experimental evidence, to be ascribed to auxin binding proteins, for example, the maize site III protein is located

at the plasma membrane and has a concentration of 20 to 150 pmol per gm fresh weight. This should meet the requirements of a polar plant hormone transport molecule (Raven et al. 1981).

With regard to the control of gene expression, auxin binding factors have been shown to activate RNA transcription rates. Roy et al. (1977) reported the extraction of two IAA binding proteins from the endosperm cells of coconut, one being located in the nucleoplasm (n-IRP) and the other located in the non-histone chromatin fraction (c-IRP). The c-IRP has two IAA binding sites, K_D's of 58 nM and 8.2 μM but the specificity of the binding sites was not established. They proposed a model (similar to that for steroid hormone receptors) in which the c-IRP in the chromatin acts as an acceptor for the IAA-n-IRP complex which then activates gene expression. The *in vitro* enhancement of the transcription rate was demonstrated in a separate experiment, although more evidence is required before this model can be accepted as a general model for plant genome activation. Rizzo et al. (1977) isolated a 'transcription factor' from soybean hypocotyl by affinity chromatography, using 2,4-dichlorophenoxyacetyl-ε-L-lysine coupled to sepharose. This linkage is in fact thought to be questionable as the carboxyl group is thought to be important in the auxin binding, however, this factor did stimulate *in vitro* RNA transcription and it has been suggested that it may be involved in the *in vivo* stimulation of soybean RNA polymerase by 2,4-D.

While attempts are being made to allocate a function(s) to the various auxin binding proteins isolated, it is of interest to note the points made by Trewavas (1976a and 1979). He states that any initial plant cell-auxin interaction need not require the mediation of a protein receptor and that the plasticity characteristic of plant development, in which induction processes can be environmental and non-specific, as well as hormonal,

requires a common coupling mechanism, which could be something as simple as the modulation of membrane permeability.

Plant hormone binding proteins are biochemically analysable entities. However the functions of these factors have yet to be elucidated, and while it would be scientifically pleasing to emulate the strides made in the research into animal hormone receptors, a direct comparison between animal receptors and putative receptors in plant tissue may well be an oversimplification.

CHAPTER FIVE

INHIBITOR STUDIES

The use of biochemical inhibitors is widely applied in the investigations undertaken into the control of gene expression. This section will relate the findings with regard to the use of protein synthesis inhibitors and a protein glycosylation inhibitor and their effect on xylem differentiation. While the primary sites of action for the inhibitors are known, there are secondary effects also, hence the results determined using inhibitors must be treated with a degree of caution. This is especially the case when positive results are the outcome of the investigations, in which instance it is difficult to determine whether the primary site of action or one of the side effects are responsible for the observed reduction. Negative results could be more generally accepted, in that they strongly indicate that the biochemical event known to be inhibited by the inhibitor is not significantly involved in the process under study.

The common protein synthesis inhibitors utilised are cycloheximide, chloramphenicol, (both of which inhibit synthesis directly) and actinomycin D (which inhibits protein synthesis indirectly). Cycloheximide inhibits the 'elongation phase' of mRNA translation, acting on the 80s ribosome. Chloramphenicol inhibits the peptidyl transferase activity of the 50s ribosomal subunit in prokaryotes and also binds to ribosomes in mitochondria and plastids, possibly acting as an inhibitor there as well. Actinomycin D is thought to bind to double helical DNA, thereby preventing

transcription of mRNA and indirectly reducing the synthesis of new gene products.

Proteins have been shown to be glycosylated by the *en bloc* transfer of an oligosaccharide moiety from a lipid-linked saccharide intermediate. Tunicamycin, the most studied of protein glycosylation inhibitors, has been demonstrated to inhibit the initial assembly of the oligosaccharide on the dolichol intermediate, thereby preventing glycosylation. (Reviewed by Elbein 1979). However not all protein glycosylations are tunicamycin sensitive, for example Davies and Delmer (1981), studying cell free protein glycosylation in *Phaseolus vulgaris*, have demonstrated two types of glycosylation reactions, one of which is tunicamycin insensitive. The tunicamycin insensitive reaction appears to be involved in terminal glycosylations, while the core glycosylation reaction involves the lipid intermediate. Other tunicamycin-like glycosylation inhibitors e.g. streptovirudin and antibiotic 204010 (James and Elbein 1980) have achieved similar results *in vivo* and *in vitro* i.e. at concentrations in which mannose and GlcNac incorporation are strongly inhibited leucine incorporation is apparently unaffected. In contrast Bacitracin, while inhibiting the synthesis of lipid-linked saccharides and glycoproteins in mung bean seedlings (Ericson et al. 1978), *in vivo* studies have shown a strong parallel inhibition of protein synthesis. Tunicamycin would appear to be the most suitable candidate for the investigation of protein glycosylation in these studies.

The use of inhibitors is a very useful form of preliminary investigation into the biochemical aspects of differentiation. The results can often produce strong indications as to the importance of certain biochemical syntheses to the cytodifferentiation process.

RESULTS

Figures 5.1 and 5.2 demonstrate the effect of increasing concentrations of the four inhibitors on the xylogenic culture of artichoke explants. The results are expressed as a percentage of the number of xylem formed in explants grown as controls i.e. without inhibitor. All four inhibitors reduced xylogenesis at varying concentrations, with tunicamycin strongly inhibiting xylem differentiation at 100 $\mu g\ ml^{-1}$ while chloramphenicol strongly inhibited at half this value (50 $\mu g\ ml^{-1}$, 150 μM), although the inhibition was marked below this concentration also. Cycloheximide inhibited xylogenesis between 5 and 10 $\mu g\ ml^{-1}$ (18 and 36 μM) while actinomycin D only fully inhibited xylem differentiation at a concentration of 20 $\mu g\ ml^{-1}$ (16 μM). Cycloheximide also reduced the growth of the explants significantly (Table 5.1) and at a concentration of 10 $\mu g\ ml^{-1}$ caused the cessation of explant growth altogether. Actinomycin D, at a concentration of 20 $\mu g\ ml^{-1}$, significantly increased the growth of the explants while the other inhibitors, at the concentrations indicated, had no significant effect on the dry mass increase of the explants during the culture period.

Figure 5.3 shows the effect of inhibitors, when present throughout the xylogenic culture period, on the time course of xylem formation. The results are expressed as a percentage of the final number of elements formed in a control culture i.e. by day 4 of culture on xylogenic medium with no inhibitor. Cycloheximide strongly inhibited xylogenesis (and presumably growth) throughout the culture period, while tunicamycin both delayed the initiation of xylogenesis and reduced the final level of differentiation. This was not simply a delay effect, as the percentage of xylem formed did not increase further on prolonged culture. Actinomycin D and chloramphenicol reduced the number of elements formed but did not delay the initiation of xylogenesis.

With cycloheximide being a powerful inhibitor it was decided to see the effect of adding this inhibitor to a system that is already undergoing differentiation. To this end explants were grown, after preculture, on a xylogenic medium and each day a sample of these explants were aseptically transferred onto a xylogenic medium containing 10 $\mu g\ ml^{-1}$ of cycloheximide. After transfer the explants were left on the new, inhibitor-containing, medium for the remainder of the culture period (Figure 5.4). Obviously, cycloheximide, at this concentration, stopped cellular processes, including differentiation, immediately on its addition.

The length of time the artichoke tubers had been stored affected the inhibitory effect of the inhibitors (Table 5.2). The inhibitory effect of actinomycin D and chloramphenicol increased on storage of the artichoke tubers (i.e. there was a greater inhibition of xylem element formation for the same concentration of inhibitor) while the inhibitory effect of tunicamycin decreased over the same period. Cycloheximide appeared to be a powerful inhibitor, probably through its effects on growth, whatever the storage period.

It has been demonstrated that the preculturing of artichoke explants enhances the level of xylogenesis on subsequent transfer onto a xylogenic medium (Chapter 3). The use of inhibitors could possibly indicate biochemical aspects of this interaction. The inhibitors were added to the various sequences of the two step culture regime to cover the permutations available (Table 5.3). The inhibitors reduce the amount of xylogenesis in the explants whatever the sequence of addition. However the actual sequence of inhibitor addition does vary the final level of xylem formation, although no obvious pattern unites the results. Actinomycin D has its maximum inhibitory effect when added only to the xylogenic medium, while chloramphenicol has its maximum inhibitory effect when added only to the preculture medium. Cycloheximide stopped xylogenesis no matter what the sequence of addition.

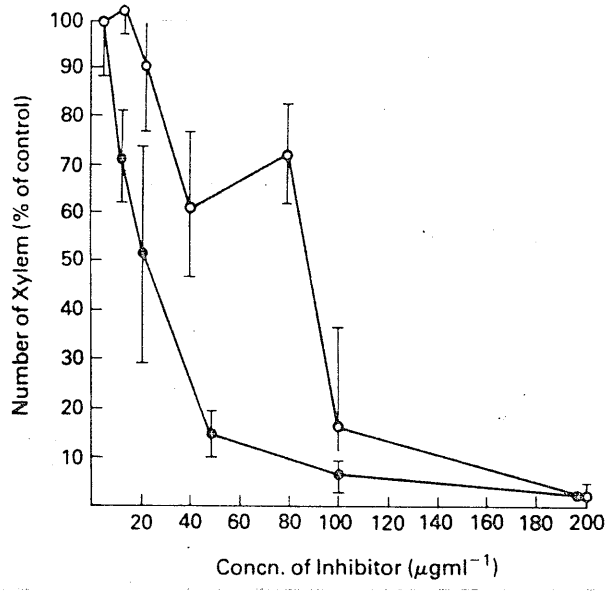

Figure 5.1 The effect of chloramphenicol (closed circles) and tunicamycin (open circles) on xylem differentiation in cultured artichoke explants grown on the xylogenic medium for 4 days. Each point is the mean ± SD of 5 explants. The artichoke tubers had been stored for 8 months.

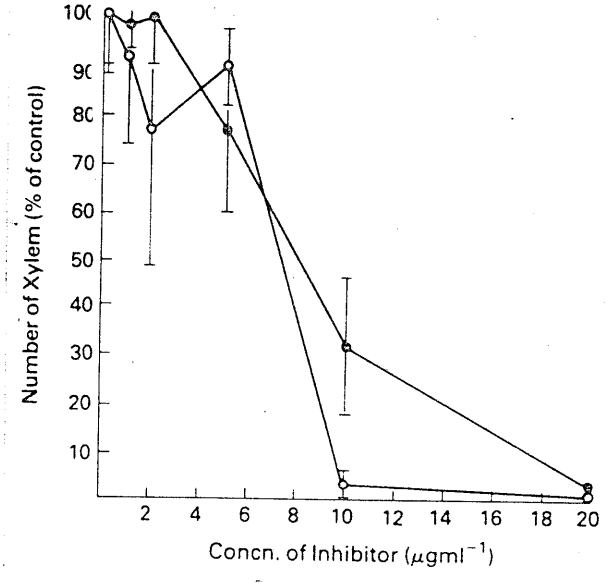

Figure 5.2 The effect of actinomycin D (closed circles) and cycloheximide (open circles) on xylem differentiation in cultured artichoke explants grown on the xylogenic medium for 4 days. Each point is the mean ± SD of 5 explants. The artichoke tubers had been stored for 8 months.

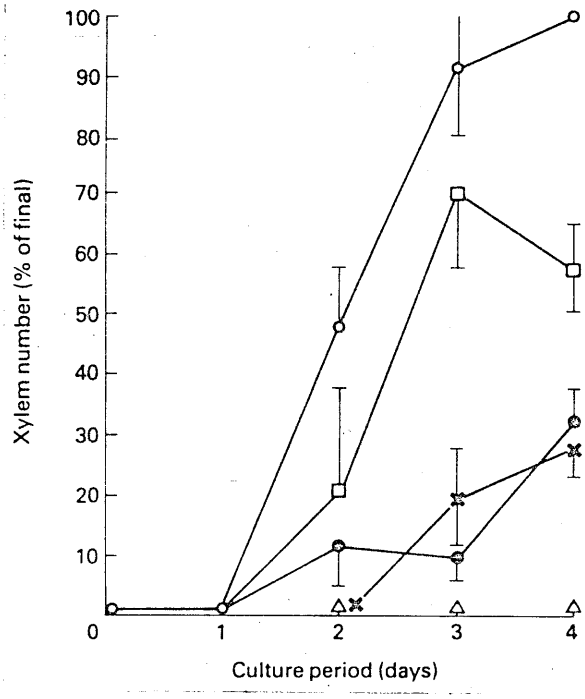

Figure 5.3 The effect of inhibitors on xylem differentiation throughout the xylogenic culture period. Control-no inhibitor (open circles), actinomycin D at 10 µg/ml (square), tunicamycin at 20 µg/ml (cross), chloramphenicol at 100 µg/ml (closed circles), and cycloheximide at 20 µg/ml (triangles). All points are the mean ± SD of 5 explants. The artichoke tubers were freshly harvested.

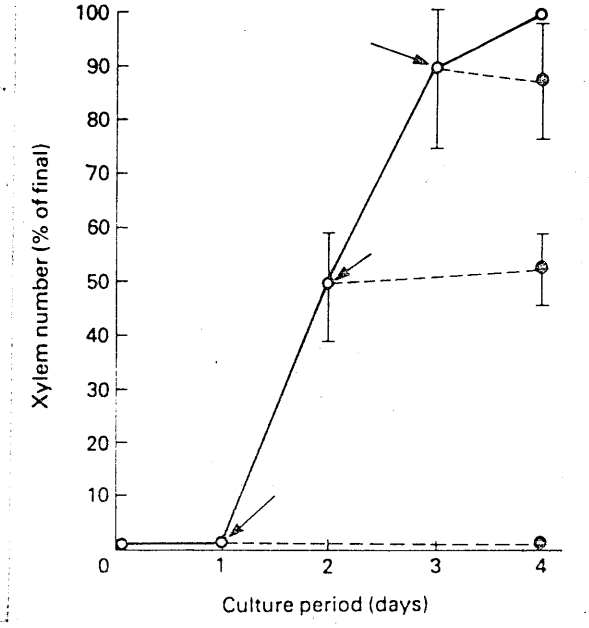

Figure 5.4 The effect of adding cycloheximide (20 µg/ml) to artichoke explants undergoing xylogenesis. The time of addition is indicated by an arrow with the final level of xylem formation with the inhibitor (closed circles) and without the inhibitor (open circles) plotted. Each point is the mean ± SD of 5 explants. The artichoke tubers were freshly harvested.

Table 5.1 The effects of inhibitors on the growth of cultured artichoke explants.

Inhibitor	Final dry mass per Explant (mg)	Increase in dry mass per Explant (mg)	Growth as a % of Control
Control (No inhibitor)	12.1 ± 2.5	4.2	100
Actinomycin D (10 $\mu g\ ml^{-1}$)	12.0 ± 1.6	4.1 (ns)	97.6
Actinomycin D (20 $\mu g\ ml^{-1}$)	15.2 ± 2.0	7.1 ($p < 0.01$)	173.8
Tunicamycin (50 $\mu g\ ml^{-1}$)	11.9 ± 1.6	4.0 (ns)	95.2
Tunicamycin (100 $\mu g\ ml^{-1}$)	11.4 ± 2.1	3.5 (ns)	83.3
Chloramphenicol (50 $\mu g\ ml^{-1}$)	13.5 ± 3.4	5.6 (ns)	133.3
Chloramphenicol (100 $\mu g\ ml^{-1}$)	14.9 ± 3.8	7.0 (ns)	166.7
Cycloheximide (5 $\mu g\ ml^{-1}$)	8.9 ± 2.1	1.0 ($p < 0.001$)	23.8
Cycloheximide (10 $\mu g\ ml^{-1}$)	7.1 ± 1.7	0 ($p < 0.001$)	0

The explants were precultured for 3 days and then transferred and grown for 4 days on a xylogenic medium with the inhibitor indicated or without inhibitors (control).

The results are the mean ± SD of 10 explants. The significance of the differences in growth was determined using the t-test.

Table 5.2 The effect of Jerusalem artichoke tuber storage on the inhibitory effect of biochemical inhibitors as inhibitors of xylogenesis in cultured explants.

Storage Period (Months)	Inhibitor	Xylem (% of control)
0	Control	100
0	Actinomycin D (10 $\mu g\ ml^{-1}$)	58.9
4	" "	60.3
8	" "	35.8
0	Tunicamycin (20 $\mu g\ ml^{-1}$)	27.8
4	" "	86.4
8	" "	90.2
0	Chloramphenicol (100 $\mu g\ ml^{-1}$)	33.7
8	" "	3.8
0	Cycloheximide (10 $\mu g\ ml^{-1}$)	0
8	" "	0

The explants were grown on the xylogenic medium for 4 days after which time the proportion of xylem elements per explant were determined and expressed as a percentage of the control. The results are the mean of 5 explants.

Table 5.3 The effect of the sequential addition of inhibitors on xylogenesis in cultured Jerusalem artichoke explants.

Inhibitor and addition sequence	Xylem (% of control)
i) Actinomycin D (10 μg ml^{-1})	
Preculture → xylogenic (control)	100
Preculture → xylogenic plus actinomycin D	58.9 ± 3.8
Preculture plus actinomycin D → xylogenic plus actinomycin D	76.3 ± 8.9
Preculture plus actinomycin D → xylogenic	82.1 ± 9.2
ii) Chloramphenicol (100 μg ml^{-1})	
Preculture → xylogenic (control)	100
Preculture → xylogenic plus chloramphenicol	33.7 ± 4.5
Preculture plus chloramphenicol → xylogenic	0.2 ± 0
Preculture plus chloramphenicol → xylogenic plus chloramphenicol	40.4 ± 15.1
iii) Cycloheximide (10 μg ml^{-1})	
Preculture → xylogenic (control)	100
Preculture → xylogenic plus cycloheximide	0
Preculture plus cycloheximide → xylogenic	0
Preculture plus cycloheximide → xylogenic plus cycloheximide	0

The proportion of xylem elements per explant was determined after 4 days culture on the xylogenic medium. The results are expressed as a percentage of the xylem formed in the control. Each result is the mean ± SD of 5 explants. (The tubers had been stored for 2 months.)

DISCUSSION

The use of biochemical inhibitors to investigate biological processes is one in which a degree of caution is required when analysing the results. However, given the knowledge of the primary site of action of the inhibitor and the awareness of the likelihood of secondary effects, it is possible for such studies to give reasonable indications about the associated biochemistry of the process under investigation. Xylem differentiation is a complex cellular transformation, presumably involving numerous intracellular biochemical modulations. Given the rigid ontology of xylogenesis, a disruption of the associated biochemistry within the cells should inhibit xylem element formation. This appears to be verified in the findings in this section.

Protein synthesis has been previously found to be a requirement for xylem differentiation (Fosket and Miksche 1966). The authors, using cultured *Coleus* stem slices, observed that 10 $\mu g\ ml^{-1}$ of actinomycin D inhibited xylem differentiation without reducing the growth of the explants, while chloramphenicol, at a concentration of 10^{-3} M, inhibited both. The results in this section, using cultured artichoke explants, showed the same finding for actinomycin D as that of Fosket and Miksche but differed in the finding with chloramphenicol, where at 10^{-4} M it inhibited xylem differentiation while, if anything, slightly increasing the growth of the explants.

The ability of cycloheximide to prevent xylogenesis depends upon either its ability to inhibit the synthesis of new key proteins required for differentiation or to inhibit the continued production of proteins essential for the ongoing cellular processes. The potency of actinomycin D as an inhibitor of xylogenesis will depend upon its ability to prevent the formation of key mRNA transcripts and the turn over rate or life expectancy of these. From the above results it would appear that protein synthesis is

a requirement for xylem differentiation (although it is difficult to separate the effect of cycloheximide on the normal processes of the cell from those associated with xylem differentiation) as is transcript production. It is interesting to note that while cycloheximide remains a potent inhibitor of xylogenesis throughout the storage time of the artichoke tubers, actinomycin D increases in potency as the tubers are stored. This could possibly be due to the fact that freshly harvested artichoke tubers contain a population of mRNA molecules (some of which are important to the differentiation process) which diminishes on storage and thereby increasing the inhibitory effect of actinomycin D.

Actinomycin D, while reducing the proportion of xylem elements formed, does not alter the general trend of xylogenesis throughout the culture period. On the other hand, cycloheximide stops xylem element formation even in a system which is undergoing xylogenesis prior to the addition of the inhibitor, however it presumably acts through its general toxic effects rather than solely at the xylogenic level.

Chloramphenicol directly inhibits protein synthesis in prokaryotes and will also inhibit protein synthesis in mitochondria and plastids. While the effect of mitochondrial and plastid metabolism on xylogenesis has not been reported, the primary effect of chloramphenicol would not be expected to inhibit xylem differentiation. The fact that it does, indicates that one or several of the secondary effects of chloramphenicol are probably inhibitory. It is worth considering the fact that chloramphenicol is frequently utilised as an antibacterial agent for *in vitro* and tissue culture studies and, that in view of these findings, it should be used with caution in future experiments. As a consequence of the inhibitory effect of chloramphenicol on xylem differentiation, it proved necessary to provide incubation conditions, for example in the radiolabelled precursor incorporation studies, as aseptic as possible

without the use of an antibacterial agent. To this end a requirement for the preclusion of infected tissue is essential and makes the two step culture regime with a preculture period particularly useful.

Tunicamycin is an inhibitor of protein glycosylation and has usually been utilised in the *in vitro* analysis of this process in animal and plant fractions. Its ability to inhibit xylem differentiation has not been previously reported, but from these studies tunicamycin has been shown to inhibit xylem differentiation and to delay the initiation of xylogenesis in cultured artichoke explants. The possible importance of protein glycosylation within the biochemical framework of events leading to the xylem vessel is indicated from these studies.

In view of the two step regime utilised in this study, a sequential addition of inhibitors has proved useful in indicating the significance of the preculture period on subsequent xylogenesis on the xylogenic medium. If the biochemical events occurring during the preculture period had little relationship to those occurring during the subsequent xylogenic phase, then the addition of inhibitors to the preculture medium alone should have little effect on the final level of xylem element formation. This makes the, possibly unjustified, assumption that inhibitor taken in to the cells during the preculture period is rapidly diminished (either metabolically or by some form of sequestration) on transfer to an inhibitor free xylogenic medium. Actinomycin D additions indicate a requirement for new mRNA synthesis in both the preculture period and the xylogenic period, as well as pointing to the possible positive role the preculture period may play in the subsequent xylem differentiation. Cycloheximide is obviously toxic to all cellular processes at this concentration, while chloramphenicol, which is thought to be acting through its secondary effects, gives a most enigmatic set of results, being inhibitory in every sequence of addition and with maximum inhibition when added only to the preculture medium. While there

is no obvious pattern to this set of results, it would appear to indicate that the preculture period contains biochemical events which are significant to subsequent xylem formation on transfer of the precultured explants onto a xylogenic medium.

In summary, a requirement for protein synthesis, mRNA synthesis and protein glycosylation has been shown for xylem differentiation in cultured explants of artichoke tuber tissue.

CHAPTER SIX

PROTEIN SYNTHESIS AND XYLEM DIFFERENTIATION

Xylem differentiation is observed, morphologically, as a controlled sequence of cytological events, terminating with a finely sculptured dead cell. Given the closely controlled cytodifferentiation sequence, one would expect an induction or activation of specific enzymes or structural proteins to precede such a formidable cellular transformation. One of the simplest definitions of differentiation is that given by Jacob and Monod (1963) which says that two cells may be considered as differentiated if while containing the same genome the pattern of proteins in each is different. With a suitable system for an investigation, the determination of the 'pattern of protein' synthesis elicited within the differentiating tissue is an obvious and interesting line of research to be undertaken. However relatively little research has been reported on this aspect of xylem element differentiation and that which has concerned itself with the gross accumulation or incorporation of radioactively labelled amino acids. No detailed analysis of the proteins present or synthesised in a large scale xylem differentiating system has been found.

Electron microscopic studies of differentiating xylem elements indirectly indicate an increase in protein synthesis in association with a large scale increase in the subcellular protein synthesising organelles. Cronshaw and Bouck (1965) observed a rise in rough endoplasmic reticulum in xylem elements of *Avena* coleoptiles while Srivastava and Singh (1972),

studying differentiating xylem vessels of corn, noted a marked change in nearly all the cellular organelles except plastids. An increase in rough endoplasmic reticulum and ribosomes was observed and they stated that the endoplasmic reticulum probably plays a role in cell autolysis.

Protein synthesis, not surprisingly, has been found to be essential for xylem differentiation e.g. in cultured *Coleus* stem slices (Fosket and Miksche 1966) where, in addition to the findings with inhibitors (Chapter 5), using ^{14}C-leucine incorporation studies, they observed an increase in the rate of protein synthesis during the first two days of the culture. This preceded the first visible signs of wound vessel formation but could equally, and probably more likely, have been associated with the wound response to the explant preparation procedure.

Simpson and Torrey (1977), using pea root cortical explants, studied protein synthesis, namely the incorporation and accumulation of ^{14}C-leucine, in two supposedly comparative cultures. One culture, containing the hormonal additives of 1 μM IAA and 5 μM kinetin, grew exponentially and produced a large number of xylem elements (see Chapter 3) while the other culture, lacking the cytokinin, demonstrated neither cell number increase nor xylem differentiation and was, as such, not a true control. A rapid turnover of protein was observed in the kinetin grown explants during the first 48 hrs of culture and was probably associated with cell autolysis and the wound recovery from the explant preparation procedure. At the time of xylem appearance in the explants it was observed that the rate of ^{14}C-leucine incorporation was less than its accumulation, suggesting that protein degradation was turned off or minimal. The significance of this to the cytodifferentiation process could not be ascertained without comparison to a suitable control explant culture.

Dudley and Northcote (1978) compared the *in vitro* translation products of mRNA isolated from suspension cultures of *Phaseolus vulgaris* grown either on a maintenance medium (in which the cells grew) or an induction medium (in which certain cells differentiated as xylem elements and phloem cells also). Several differences in the relative amounts of certain polypeptides were noted, with the general findings being that, the cells in the induction medium coded for relatively large amounts of fewer polypeptides whereas the cells in the maintenance medium coded for a large number of polypeptides all present in fairly equal amounts. It would not be possible to correlate any of the above changes with xylem differentiation as the level of xylogenesis is very low in cultures of this tissue.

Utilising different techniques, that of immunoelectrophoresis and other immunochemical studies, Khavkin et al. (1980) have studied cyto-differentiation in intact and cultured tissues of maize. A group of antigenically distinct proteins characteristic of the vascular cylinder of maize seedlings were determined. The stelar antigens were recorded in 13 to 15 day old developing embryos and several were found in embryo-derived callus tissues and stem derived cell suspension cultures. Several minor stelar antigens characteristic of the completed stele were also analysed but it was not possible to designate any of the antigens specifically to developing xylem elements.

RESULTS

The protein studies were undertaken in an attempt to determine quantitative and qualitative changes in separated polypeptides during xylogenesis. ^{14}C-lysine was incorporated into artichoke explants grown either on the preculture medium, the xylogenic medium or the control medium. The extracted proteins were fractionated into cytosol located and particulate

located proteins and analysed by one and two dimensional acrylamide gel electrophoresis.

i) One Dimensional Analysis

Initially electrophoretic gels were simply stained with coomassie blue to visualise the polypeptide bands. However no changes were noticeable and therefore radioactively labelled amino acids were utilised instead to identify those polypeptides being synthesised during the culture period. Incorporation was studied in artichoke explants grown on the preculture, xylogenic and control media, therefore for ease of comparison the fluorographs (Figures 6.1 and 6.2) are laid out such that (a) is the preculture, (b) is the xylogenic culture and (c) is the control culture. Each track on the fluorographs (containing 100 μg of protein) represents proteins from one day of the culture period and each fluorograph is representative of several gels run from each experiment. (The xylogenic culture experiment was duplicated).

Figure 6.1 shows the fluorographs of the cytosol located polypeptides separated by gel electrophoresis. The most noticeable feature of the polypeptide profile of explants grown on the preculture medium is that there is virtually no incorporation on day 0 (i.e. culture preparation and excision), except for two bands at MWr 66 kdal and MWr 60 kdal. (The tubers were stored at 4°C prior to the culture preparation and were still apparently dormant.) Total ^{14}C-lysine incorporation is greatest on day 1, when many more bands appear on the fluorograph. On day 2 of the preculture period a band, marked c, of MWr 41 kdal, appears for that day only.

Figures 6.1(b) and (c) enable a direct comparison of the polypeptide profiles present in the cytosol fraction of explants grown on the xylogenic and control media respectively. The incorporation pattern in (b) remains

constant throughout the xylogenic period, although total incorporation decreases by day 5 when growth has almost ceased. In comparison, in (c), the specific incorporation of ^{14}C-lysine is much greater on days 1 and 2 and very much lower on day 5 (no growth). Band b, MWr 20 kdal, increases over days 1 and 2 but disappears after that, and band a, MWr 15 kdal, is prominent throughout the control period but hardly noticeable in the xylogenic culture. Band d, MWr 132 kdal, is present in explants cultured on both media but while remaining faint in the xylogenic culture it disappears on day 4 of the control culture.

The polypeptide profiles of the particulate fraction are harder to analyse (Figure 6.2) due to the higher background exposure on the fluorographs. This is due to the fact that there is a greater degree of heterogeneity of polypeptides, either due to the presence of a much larger number of individual polypeptides or to the presence of incompletely synthesised polypeptides located in this membranous fraction. Only one band was apparent on day 0 of the preculture period, this had a MWr of 66 kdal and was identical to one of the bands on day 0 in the cytosol fraction. The general trend of incorporation was similar to that in the cytosol fraction with only one band, d of MWr 132 kdal, obviously changing, diminishing on day 4 of the culture period. Band d in the particulate fraction comigrated with band d in the cytosol.

ii) Two Dimensional Analysis

Analysis of the *in vivo* ^{14}C-lysine labelled polypeptides of cultured artichoke explants by two dimensional denaturing acrylamide electrophoresis demonstrated a much enhanced resolution of the labelled products than was available with the one dimensional SDS-PAGE. While few changes were observed in the labelled polypeptide profiles extracted from the explants

grown on the preculture, xylogenic and control cultures with a one dimensional separation, many more alterations, both quantitative and qualitative, were observed due to the greater resolution of the labelled products using two dimensional analysis.

The polypeptide 'spots' of note or those to be discussed in this section are labelled with an asterisk or a numbered arrow. The former refers to polypeptides seen to be present within the xylogenic culture but absent from the control cultured explants (and vice versa) for a given day, i.e. a direct comparison of the polypeptides present (synthesised) in the xylogenic compared to the control culture. The asterisk is placed adjacent to the polypeptide of interest, while the same location on the comparable fluorograph is left blank. The polypeptide spots marked with a numbered arrow refers to changes in that polypeptide within that culture over the culture period i.e. a trend of change within an individual culture. (Polypeptides present within comparable fluorographs which are the same, as judged by location, are numbered the same.)

The changes analysed within the two dimensionally separated polypeptide gels will be discussed with reference to the preculture period then between those of the xylogenic and the control cultured explants.

In the preculture period (Figure 6.3) very little ^{14}C-lysine was incorporated into the cytosol fraction on day 0, as was the case in the one dimensional gels, being similarly due to the dormant state of the artichoke tuber tissue. Only two polypeptides are apparent and they are very faint. In contrast, on day 1 of the preculture period, there is a dramatic increase in the number of polypeptide spots apparent, demonstrating also the much enhanced resolution when compared to the same fraction sample analysed by one dimensional gel electrophoresis. Many of the spots, apparent on day 1 of the preculture period, disappear or fail to be

resolved over the remainder of the preculture period and therefore only the more prominent fluctuations will be noted.

Polypeptide spot 6 and groups of closely located polypeptides, marked 2, 3 and 4 are relatively intensely labelled on day 1 but diminish rapidly by day 3 of the preculture. A group of polypeptides, marked 1, disappear or fail to be resolved on day 2 only to reappear on day 3, while polypeptide 5, which is intensely labelled on day 1, disappears completely after this.

The trend of ^{14}C-lysine incorporation into the xylogenic and control cultured artichoke explant cytosol fractions (Figure 6.4 a and b) was similar to that of the preculture period, and reflected the general trend of incorporation observed with the one dimensional gels, i.e. an increase in incorporation on day 1 of the xylogenic culture and days 1 and 2 of the control culture, followed by a decline over the remaining period. This is reflected in an increase in the number and intensity of polypeptide spots apparent on day 1 of the xylogenic and control cultured explants when compared to day 3 of the precultured explants (while noting that 200 µg of protein was applied to every IEF gel in the first dimension).

Polypeptides 7 and 8 remain relatively intensely labelled throughout the xylogenic and control cultures, while a group of four polypeptides, marked 17 were intensely labelled on day 1 of the xylogenic period, fluctuating and declining in intensity thereafter. This same group of proteins, also present in the precultured explants, was relatively weakly labelled on day 1 of the control cultured explants, disappearing (or possibly migrating with the marker dye) on day 2 and then reappearing, although poorly resolved, on day 3 and 4 of the control culture period. Polypeptide 4, which was intensely labelled on day 1 of the preculture period, declining after this, reappeared on day 1 of the control and

xylogenic cultures, although more intensely labelled in the control cultured explants. This spot again declines in intensity over the remaining xylogenic and control culture periods.

Polypeptide spots 9 and 10 and a group of faintly labelled polypeptides, marked 16, are apparent on day 1 of the xylogenic period but disappear after this. Similarly in the control cultured explants, polypeptides 11, 12 and 13 are resolved on day 1 again disappearing after this.

A direct comparison between the fluorographs of polypeptides present in explants undergoing xylogenesis and those simply growing are displayed in Figure 6.4a and b where the same day for each culture are situated adjacently. The asterisks mark resolved polypeptides which are absent in the opposing fluorograph. Many differences are apparent between the explants growing on the xylogenic medium and explants growing on the control culture. While many of the asterisked polypeptides are faintly labelled, especially on day 2 of the control culture, there are also differences noted in relatively intensely labelled polypeptides. The comparison of polypeptides present in the xylogenic and control cultured explants was only really possible over days 1 and 2 of the culture periods, as the intensity of the separated polypeptides declined rapidly over days 3 and 4, making detailed comparisons meaningless. An attempt to load more protein onto the IEF gels for the cytosol fraction from explants grown over the days of little radioactively labelled lysine incorporation proved ineffective. Protein loads greater than 200 μg caused retention at the IEF gel interface. None the less, as the maximum rate of xylem differentiation occurred over days 2 and 3 of the xylogenic culture period, the xylogenic associated events should be present in the intensely labelled fluorographs shown in Figures 6.4 a and b.

Figures 6.5, 6.6a and 6.6b show the two dimensional separation of proteins located in the particulate fractions of artichoke explants grown on the preculture, xylogenic and control culture media respectively. It is evident that the two dimensional analysis of the particulate located polypeptides greatly enhances the resolution afforded by the one dimensional analysis, for example compare Figure 6.6b, days 1 and 2 with the same fraction analysis in Figure 6.2c, days 1 and 2. While the enhancement is evident, it has proved much more difficult to analyse the fluorographs generated by the two dimensional analysis of the particulate located polypeptides than was the case for the cytosol located polypeptides (Figures 6.3 and 6.4). The reasons for the lack of clarity in the separated particulate located labelled polypeptides are probably several fold. Firstly, protein heterogeneity in this fraction (probably due to the presence of partially completed proteins synthesised in the membranous fraction) is relatively high and manifests itself as a background darkening. Secondly, the particulate located polypeptides have a greater tendency to 'streak' thereby making analysis difficult. The streaking is possibly due to both the natural protein charge heterogeneity in the particulate fraction proteins and the interactions of the proteins and lipids in this fraction. Thirdly, the tendency of the particulate located proteins to be retained at the IEF gel interface means that, at least, certain proteins do not migrate to their isoelectric point in the first dimension and are therefore separated only one dimensionally on transfer of the IEF gel onto the slab gel for the second dimension. This was particularly apparent in the two dimensional separation of the particulate fraction in the precultured explants.

Several attempts were made to improve the resolution and to reduce the streaking of the spots and the retention of the proteins at the IEF separation of the particulate proteins. A delipidation of the fraction with chloroform:methanol (2:1) failed to improve the resolution and caused

the loss of certain polypeptide spots present prior to the delipidation process. The reverse loading of the proteins onto the acidic end of the IEF gels, as opposed to the normal loading at the basic end, similarly proved ineffectual. Finally, the most recent method of two dimensional separation, using non-equilibrium pH gradient gel electrophoresis (NEPHGE) in the first dimension (utilised usually to prevent cathodic migration and loss of basic proteins in the first dimension) (O'Farrell et al. 1977) was also undertaken but without improvement.

Together with the difficulties encountered with the two dimensional analysis of the particulate located proteins, a difficulty was also met in trying to equate the minor changes in polypeptide profile observed at the one dimensional level within the much larger fluctuations seen at the two dimensional level. Firstly, for any one position within the one dimensional track there are often several polypeptides positioned at the equivalent MWr location within the two dimensional gel. Secondly, although molecular weight markers can be applied to the two dimensional gels, the application must be at either side of the IEF gel, hence very near the edge of the gel. It has been observed that polypeptides migrate more slowly at the edge of a gel causing difficulty in the assignment of molecular weights to the two dimensionally separated polypeptides. For these reasons the changes in polypeptides at the two dimensional resolution are discussed in terms of relative position rather than by the assignment of a molecular weight.

In view of the difficulties outlined above, it is probably more pertinent to discuss the general findings relating to the changes in the particulate located proteins as assessed by two dimensional analysis. In the preculture period (Figure 6.5) no labelled proteins were apparent on day 0 while many polypeptides were resolved on day 1. Many of these polypeptides declined in intensity or failed to be resolved over the remaining culture period. (The very high retention of protein on days 2

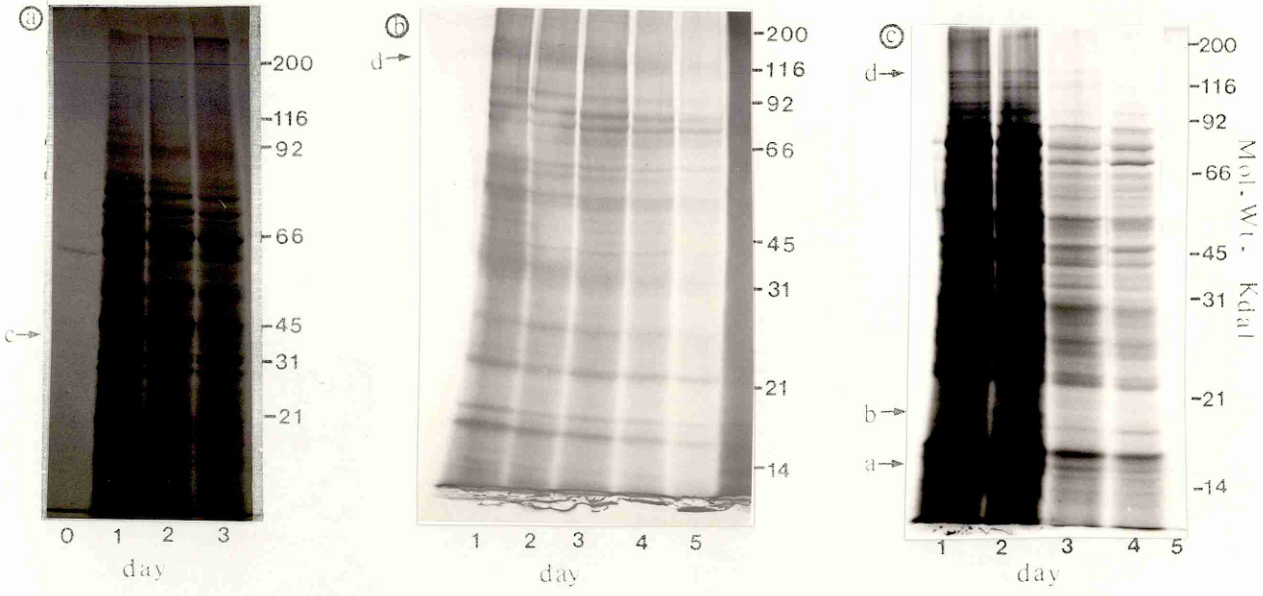

Figure 6.1 Fluorographs of the artichoke homogenate cytosol located, ^{14}C-lysine labelled, polypeptides separated by SDS-PAGE for each day of (a) preculture, (b) xylogenic culture and (c) control culture. Each track contained 100 µg of protein. Molecular weight markers migrated as indicated.

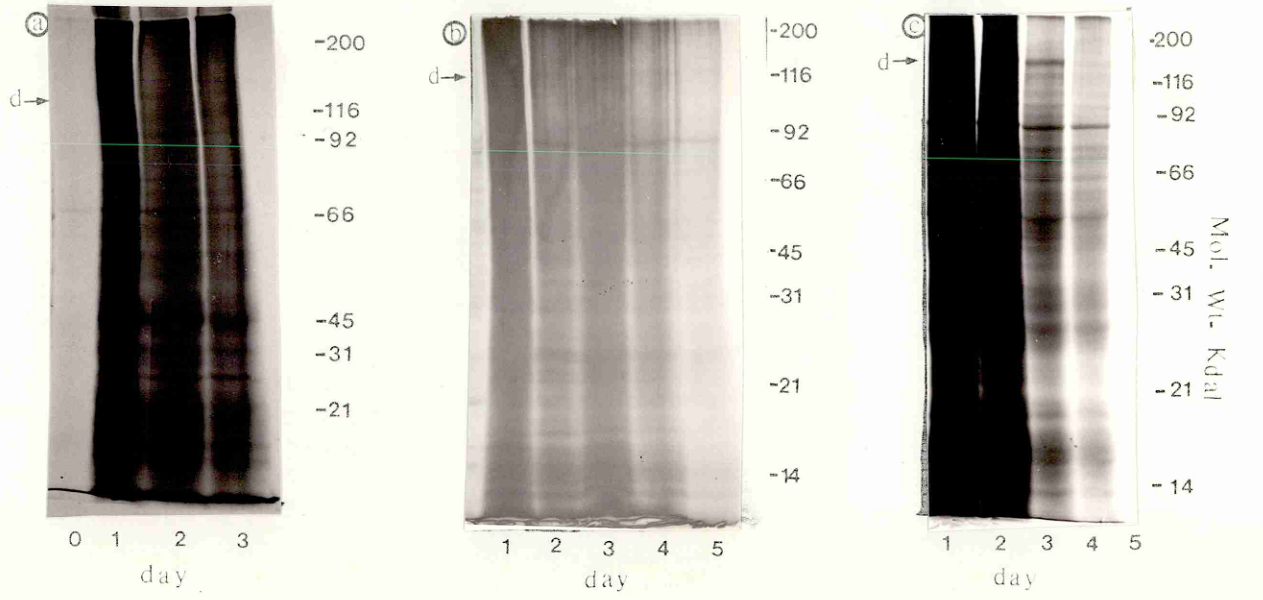

Figure 6.2 Fluorographs of the artichoke homogenate particulate located, ^{14}C-lysine labelled, polypeptides separated by SDS-PAGE for each day of (a) preculture, (b) xylogenic culture and (c) control culture.

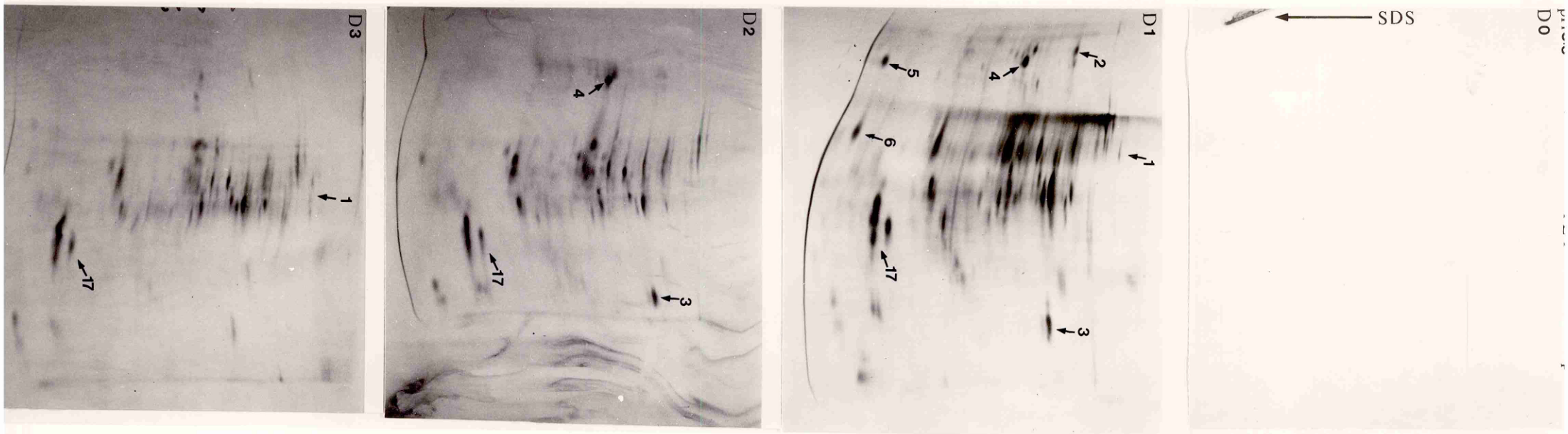

Figure 6.3 Fluorographs of ^{14}C-lysine labelled polypeptides, located in the cytosol fraction for each day of the preculture period, resolved by two dimensional gel electrophoresis. 200 µg of extracted protein was applied to the first dimensional gel for each day of the culture period. Second dimension gels were 10% acrylamide.

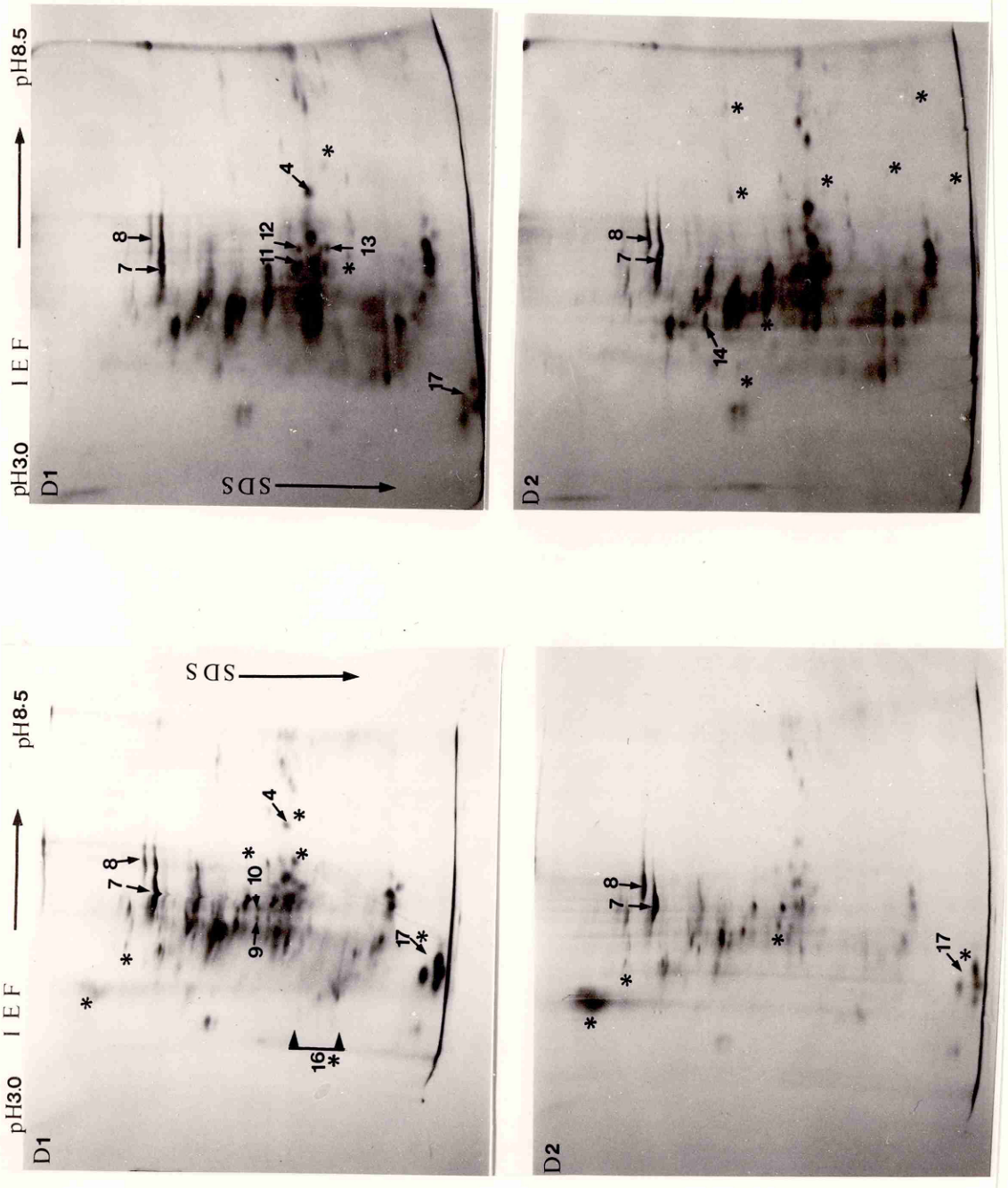

Figure 6.4 Fluorographs of ^{14}C-lysine labelled polypeptides, located in the cytosol fraction for each day of (a) the xylogenic culture period and (b) the control culture period, resolved by two dimensional electrophoresis.

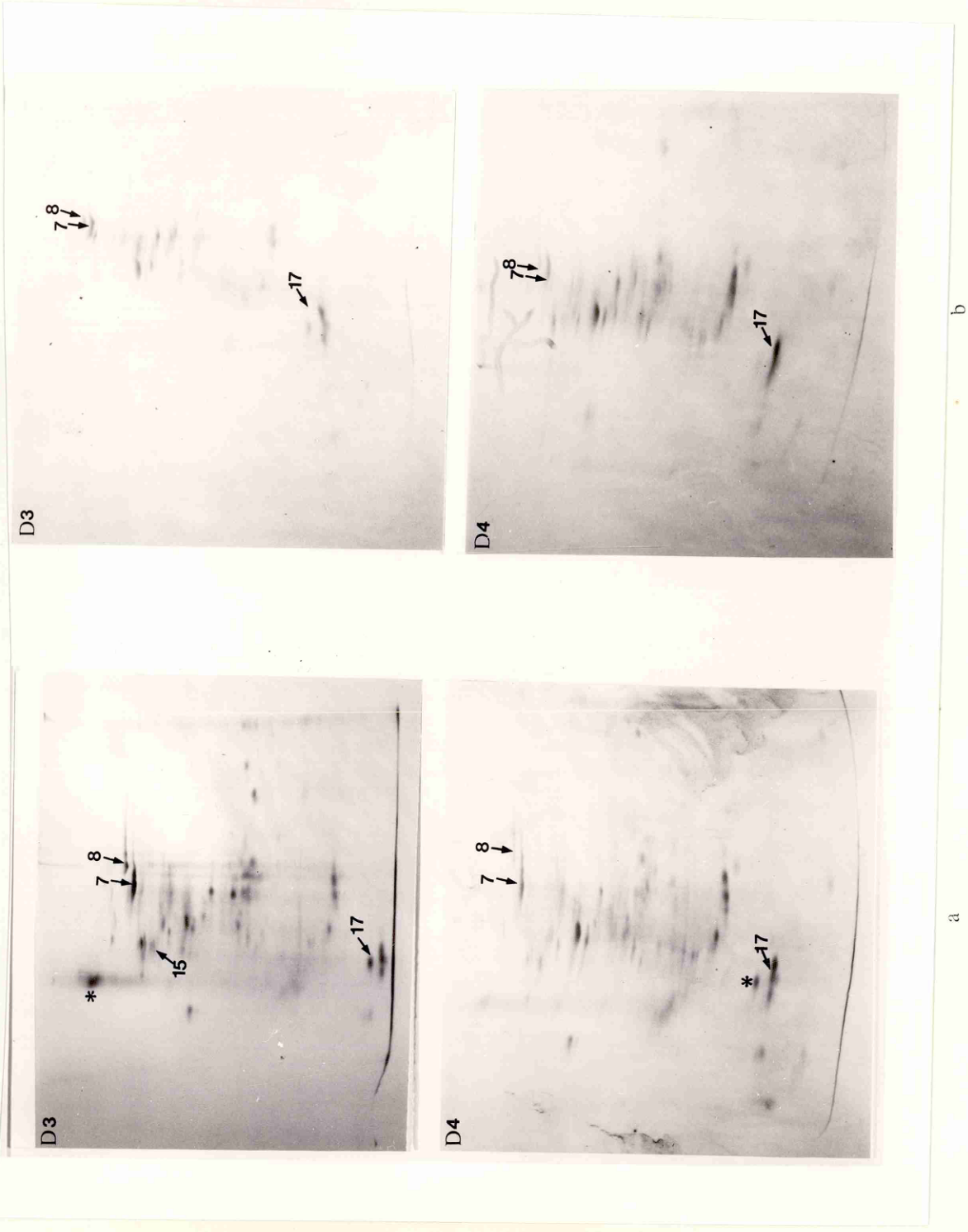

Figure 6.4 cont.

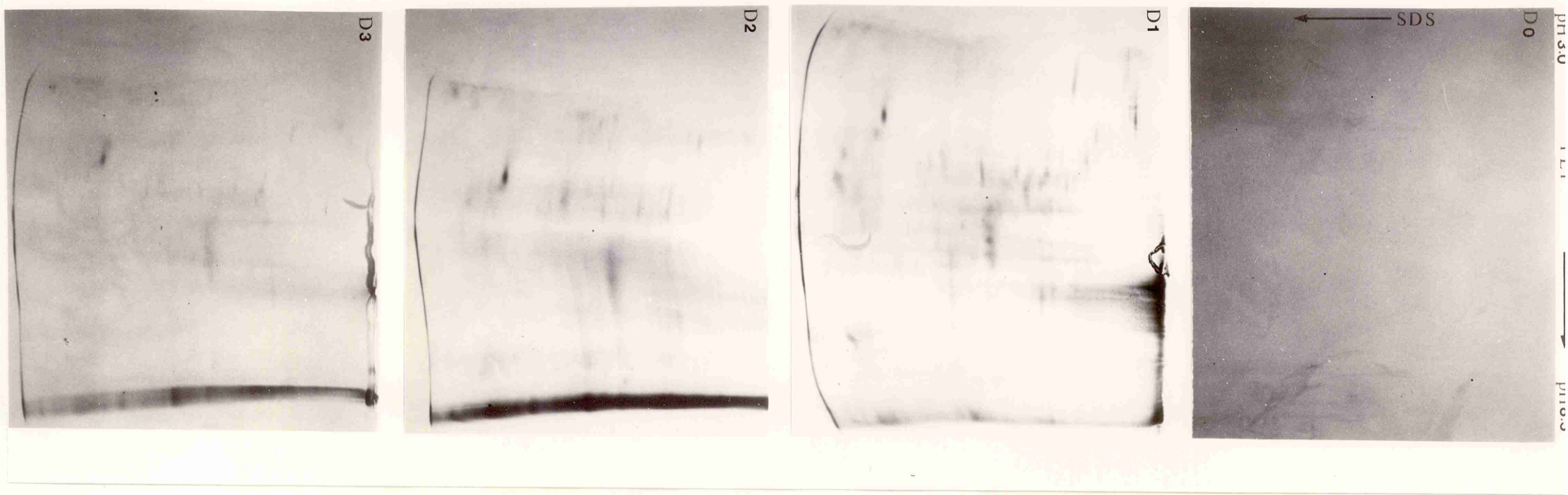

Figure 6.5 Fluorographs of ^{14}C-lysine labelled polypeptides, located in the particulate fraction for each day of the preculture period, resolved by two dimensional electrophoresis.

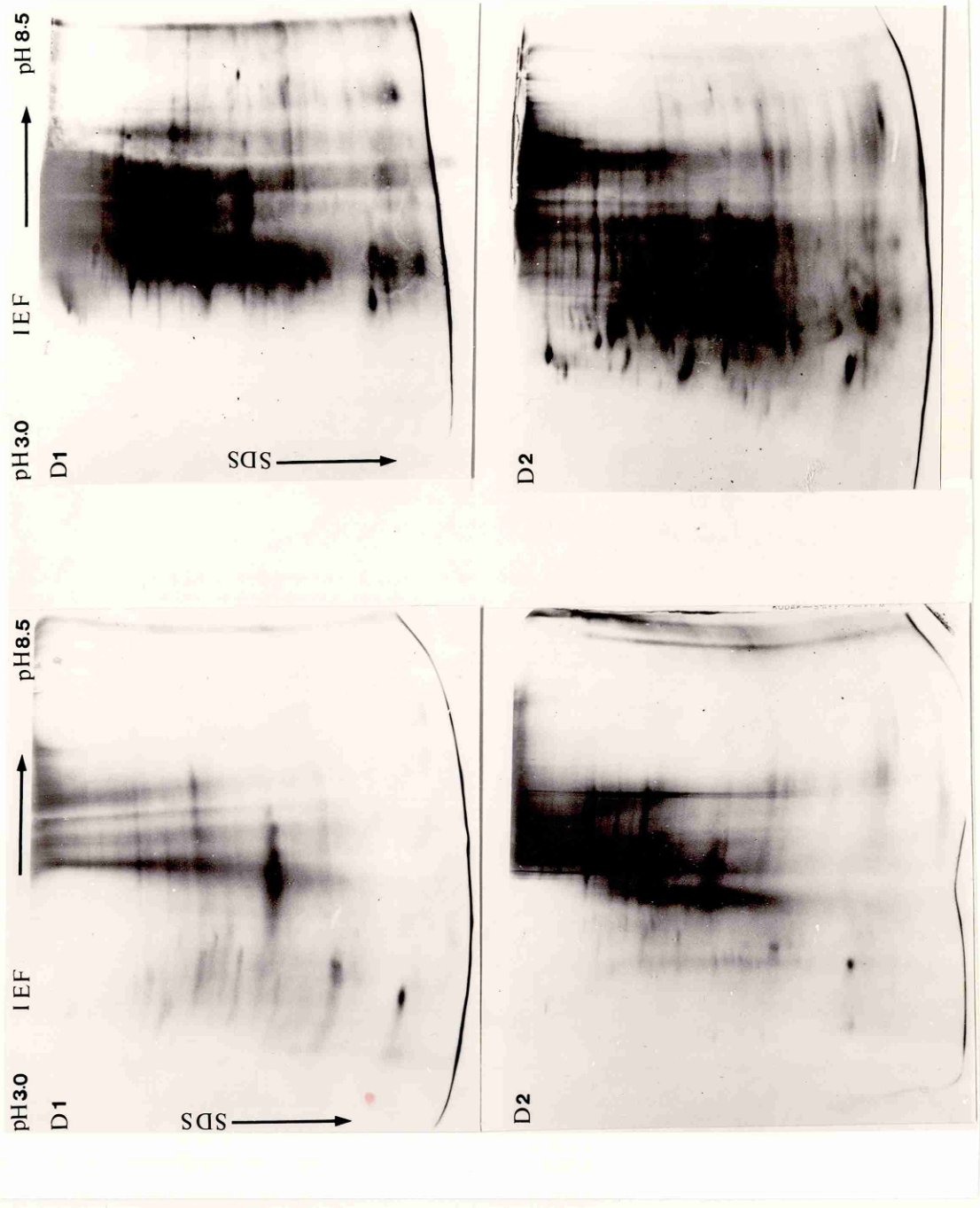

Figure 6.6 Fluorographs of ^{14}C-lysine labelled polypeptides, located in the particulate fraction for each day of (a) the xylogenic culture period and (b) the control culture period, resolved by two-dimensional electrophoresis.

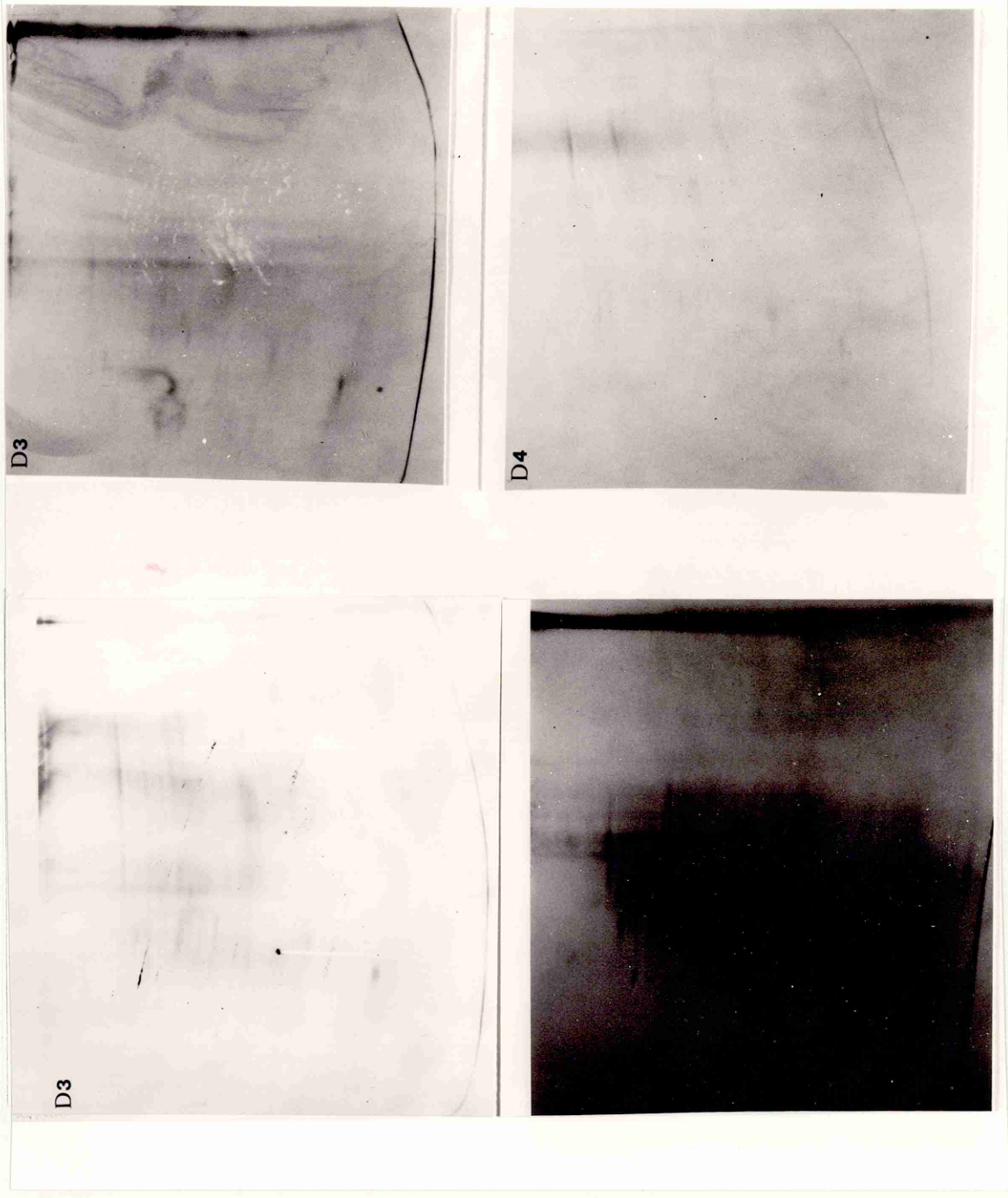

Figure 6.6 cont.

and 3 of the preculture period was evident.) A comparison of the fluorographs developed using particulate located proteins from the xylogenic and the control cultured artichoke explants demonstrate many differences (Figures 6.6a and b). While the polypeptides apparent on days 1 and 2 of the xylogenic cultured explants are very similar and also similar, although less intense, to that of the control cultured explants on day 1, there is a much larger number of polypeptides apparent on day 2 of the control culture. The fluorographs declined in intensity after this time for both cultures.

The major differences in labelled particulate located polypeptides, as resolved by two dimensional gel electrophoresis, are found to be associated with the artichoke explants simply growing, while the explants undergoing xylem differentiation present a much simpler polypeptide profile.

DISCUSSION

Cytodifferentiation is by necessity a very ordered process, in which one stage follows another within some constraining form. It is self evident that structural and functional differences between cell types, originating from the same meristem in the intact plant or meristemic regions in cultured tissues, must have a molecular basis and it is probably the ultimate aim of developmental biologists to understand differentiation in these terms.

Protein synthesis has been demonstrated to be essential for xylem formation and therefore the polypeptide patterns generated by fractionated differentiating cells is an obvious line of investigation. However, coomassie blue staining of the separated polypeptides in the one dimensional gels indicated no obvious changes in the polypeptide profiles during

xylogenesis. The reasons for this could be several; firstly this may be a genuine reflection of the cellular events, in that there is no new synthesis of xylogenic associated proteins. More realistically those biochemical events occurring may be masked by the quantitatively predominant plant structural proteins or the resolution of the minor polypeptides using coomassie blue may be inadequate. The utilisation of *in vivo* radiolabelled precursor studies and the enhanced resolution offered by the two dimensional separation of the labelled polypeptides overcame many of the problems outlined above, with regard to protein synthesis.

The discussion of the radiolabelled polypeptide profiles results will be grouped into those changes thought to be associated with the wound response, growth response and xylogenic response of the artichoke tissue respectively. Utilising the two step culture regime detailed and discussed previously it should be possible to separate the biochemical events associated with the wound response and the growth response of the tissue from those associated with the xylogenic response. The events singular to the preculture period probably represent components of the wound response of the cultured tissue, while those events common to the xylogenic and control culture periods should represent components of the growth response. The biochemical events singular to the xylogenic culture should represent the key modulations associated with xylem differentiation and those singular to the control culture could possibly relate to xylogenic inhibitory factors. While it is not possible to definitely couple a biochemical event to a distinct cellular event at this preliminary level of investigation, it is possible to obtain a strong indication for further investigation, if the observed event occurs at the time of xylogenesis i.e. on days 2 and/or 3 of this xylogenic culture. That is not to say that the events occurring during the preculture period are not integral to the subsequent xylem differentiation, as the similarity between xylem elements formed in tissue

culture and the wound vessel members formed in intact plants is noteworthy. However if this is the case, then the components related to the xylogenic events, which are synthesised as part of the wound response, must be quickly depleted when explants are transferred to the control medium, on which few xylem are formed.

The changes in radiolabelled polypeptides witnessed during the preculture period can be considered to encompass, at least, the biochemical modulations occurring in response to plant tissue wounding. To place the findings of this report within the field of plant wound biochemical knowledge, it is first necessary to discuss other research findings with regard to protein synthesis and enzyme activity modulations, some of which include the *de novo* synthesis of enzymes which should be reflected in the fluorographs for the preculture period. The cellular wound response (a biochemical and physical reaction to slicing, infection or chemical treatment) of plant tissues depends upon whether the cell is physically damaged, in which case it dies, or is adjacent to a severed cell, in which case it responds to the wounding 'stress'. Wounding induces specific changes in the activity of most enzymes, the majority of which increase in activity, although a few remain unchanged and a very few actually decrease in activity. The initial responses of wounded potato tuber tissue have been investigated (Kahl and Wielgat 1976), the primary response being a vigorous synthesis of rRNA for up to 24-48 hours post-wounding and a transition of the cytoplasmic located ribosomal monosomes into polysomes, indicating an activation of protein synthesis. The activation of protein synthesis can continue for up to 3 days involving the *de novo* synthesis of structural and enzymic proteins. In the dormant tissue, prior to wounding, there is no protein synthesis occurring, although there are sufficient ribosomes present in the cytoplasm only requiring the addition (*in vitro*) or the presence (*in vivo*) of mRNA, to be activated. Various experiments on plant RNA polymerases, present in the dormant tissue, show them to be inactive even though there are 'unblocked'

genes located in the dormant tissue chromatin, as evidenced by the addition of exogenous bacterial RNA polymerases. The wounding therefore induces the activation of RNA polymerses followed by ribosomal construction and formation into polysomes when linked to the newly synthesised mRNA, ultimately inducing a large scale increase in cellular protein synthesis.

Most enzymes increase in activity on wounding, for example in Jerusalem artichoke tuber tissue acid invertase and ascorbate oxidase (Edelman and Hall 1965), cinnamic acid hydroxylase and NADPH-cytochrome P-450 reductase (Benveniste et al. 1977) have all been demonstrated to increase in activity on wounding, however the mechanism of activitation is not known. The activity increase may be due to an activation (e.g. covalent modification) of pre-existing enzymes or the *de novo* synthesis of the protein, both of which have been shown to occur in plant tissues. In sweet potato tissue, Matsushita and Uritani (1975) demonstrated the wound induced increase in activity of PAL and acid invertase which was inhibited by cycloheximide and actinomycin D (demonstrating a requirement for transcription and translation) while the increase in peroxidase activity was inhibited by cylcoheximide but not by actinomycin D (which did reduce RNA synthesis by 60-70%). The latter finding could be explained in several ways; i) the peroxidase mRNA may not be inhibited by actinomycin D, ii) the template site for peroxidase may be resistant to actinomycin D, or iii) the mRNA for peroxidase may be preformed prior to the wounding.

Enzyme increases in activation peak at a species specific time after slicing e.g. glucose-6-phosphate dehydrogenase activity in potato peaks 2 days after the initial wounding (Muto et al. 1969), while PAL activity peaks at 15 hours (Tanaka and Uritani 1977) and RNase activity at 24 hours post-wounding (Sucher et al. 1982) in sweet potato tissue.

A detailed analysis of proteins synthesised during the wound response has not been published, although the detailed analyses of isoenzyme patterns have. Borchert (1977) studied the isoperoxidase patterns, formed by starch gel electrophoresis in extracts of potato tuber tissue after wounding. He observed that the patterns of isoperoxidases could be correlated with the wound response and even with the wound induced cyto-differentiation (including vascular differentiation). He considered that the pattern of isoperoxidases could be utilised as a marker for wound induced differentiation. Other isoenzyme patterns have been shown to alter during the wound response; peroxidase isoenzymes (again) (Zimmermann and Rosenstock 1976), glucose-6-phosphate dehydrogenase isoenzymes (Muto et al. 1969) and phosphoglucomutase (Kahl and Stegemann 1973), all in potato.

The reasons for the wound response in plant tissues, as well as being a simple chemical reaction to the physical disturbance caused by wounding e.g. intracellular compartmental mixing, could also be a role in the protection of the wounded plant organ from infection. This has been argued to be the role for the wound induced increase in the activity of the enzymes involved in polyphenylpropanoid metabolism (Rhodes and Woolerton 1978). This can cause the accumulation of simple phenols such as chlorogenic acid or polyphenolic compounds such as lignin, which could act as a barrier to the spread of infection.

The preculture period, of the two step regime detailed in this report, was initially developed to allow for the detection of infection prior to the xylogenic or control culture of the artichoke explants. The 3 day preculture period has also proved to be useful in both providing a system for the detailed study of the wound response of the excised tissue, as an investigation in its own right, and more importantly, in this study, as a means of separating the biochemical events occurring during the wound

responses from those occurring during growth or xylem differentiation. This study has demonstrated a great many changes in the pattern of polypeptide labelling during the wound response, especially at the high resolution offered by two dimensional electrophoresis of the proteins present in the cultured tissues. However it has not been possible to identify the polypeptides, in what is a very preliminary study of the process.

The findings in this section show similarities to the general findings of others working solely on plant tissue wounding biochemistry. The stored artichoke tuber tissue is, in essence, a dormant tissue, although a very low level of protein synthesis has been shown to be occurring either in the stored tubers or very soon after the initial excision of the tissue, as evidenced on day 0 of the preculture by the presence of two ^{14}C-lysine labelled polypeptides in the cytosol fraction and one polypeptide in the particulate fraction after electrophoresis. Wounding (tissue excision and culture preparation) induced a large scale increase in protein synthesis, shown in the one dimensional and, especially, the two dimensional analysis of the labelled polypeptides, as a very large increase in the number of polypeptides in the fluorographs of the cytosol and particulate fractions from day 1 of the preculture period. After this peak of protein synthetic activity the labelling of the polypeptides declined over the remaining preculture period. Certain of the polypeptides do not just follow the general decline in labelling but demonstrate individual labelling trends, possibly indicating a more prominent role within the numerous events occurring throughout the preculture period which are grouped together and called the 'wound response' of the tissue.

The role of the polypeptide changes shown to occur during the preculture period and which have been designated the wound responses of the cultured artichoke tissue cannot be commented upon in detail in this preliminary

investigation, however it would be interesting to link the earlier work on enzyme induction and isoenzyme pattern formation during the wound response with the detailed analysis available by utilising a two dimensional separation of the cellular contents throughout this phenomenon.

Many attempts have been made to correlate the synthesis of specific proteins with an observable change in the state of a plant cell. Of the two remaining processes to be discussed in this section, growth and xylogenesis, investigations into the identification and quantification of proteins associated with the initial induction of plant growth (or elongation) by auxins and the maintenance of growth within plant tissues have certainly predominated. The primary response of plant tissues to the addition of auxin occurs within the first 15 minutes and much speculation has surrounded the role of putative growth associated proteins (termed Growth Limiting Proteins; GLP) and auxin induced growth. To place the findings of this report within the context of present day knowledge of the biochemistry of plant growth, it is necessary to present a discussion relating to protein synthesis and plant tissue growth.

A preliminary investigation of GLP in auxin-induced elongation in Lupin hypocotyls (Penny 1971) utilised the inhibitory effect of cycloheximide on protein synthesis. The author concluded that the initial phase of auxin action did not depend upon protein synthesis, which was however required for the maintenance of growth. Patterson and Trewavas (1967) observed changes in protein synthesis in subapical sections of etiolated peas induced by the addition of IAA. Utilising a dual labelling technique (which demonstrates differences in protein synthesis between a plant tissue undergoing a specific process and a comparable control, one of which is labelled with a tritiated amino acid and the other with a ^{14}C-labelled amino acid. The ratios of the two labels indicate a difference in protein synthesis when a peak or trough in the ratio occurs.) and column chromatography

(hence a rather limited resolution), changes in protein synthesis were noted. The greatest changes were observed in the particulate located proteins (which included the nuclear material), the alterations induced by the addition of IAA being susceptible to the addition of actinomycin D (10 $\mu g\ ml^{-1}$). A further attempt to unravel the association of GLP with cell elongation, in oat coleoptile utilising dual labelling and the greater resolution offered by one dimensional gel electrophoresis, failed to identify a close relationship between the two (Bates and Cleland 1980). While agreeing with Penny's earlier conclusion, that GLP synthesis was required for continued auxin induced elongation, they determined that GLP synthesis was independent of auxin and that GLP have a very rapid half life (previously determined at 12 to 17 minutes by Cleland 1971).

The much greater resolution of synthesised polypeptides afforded by two dimensional gel electrophoresis was utilised by Zurfluh and Guilfoyle (1980) in an attempt to identify GLP. The ^{35}S-methionine labelled polypeptides synthesised in soybean hypocotyl segments on addition of 2,4-D were analysed and a change in the polypeptide pattern was noted. However the length of time required for the *in vivo* labelling of polypeptides, to achieve sufficient radiospecificity for analysis, prevented their ability to detect rapid and early changes associated with the addition of auxin. To overcome this problem Zurfluh and Guilfoyle (1982) investigated the changes in the population of polyadenylated mRNA present in the soybean hypocotyl segments after the addition of auxin. The extracted mRNA's were analysed by translation in a cell free system (wheat germ and reticulate lysate systems) in the presence of ^{35}S-methionine followed by two dimensional analysis. The authors suggested that this was a marked improvement on the *in vivo* labelling method in that it allowed the study of the population of polypeptides synthesised at discrete and very short time intervals after auxin addition. The possible limitation of the method is the reliability or faithfulness of the *in vitro* synthesis of polypeptides

as a representation of the *in vivo* situation. They concluded that the levels of translatable mRNA were increased, for at least 10 products, by the addition of auxin (IAA and 2,4-D) and that the induction was rapid, less than 15 minutes. The addition of actinomycin D rapidly decreased the auxin induced *in vitro* translatable products, the mRNA having a short half life, 1.0 to 1.5 hours.

Similar work by Theologis and Ray (1982), using pea stem tissue and a two dimensional analysis of the *in vitro* translatable products of extracted poly-A-mRNA, determined an increase in 3 polypeptides, 20 minutes after the addition of IAA. No changes were noted at 10 minutes or less. They concluded that there is an early, highly selective auxin regulation of mRNA amounts or activation, becoming progressively more extensive with time.

In summary, the group of proteins called GLP have been shown to be a requirement for continued auxin induced growth elongation of plant cells. The proteins analysed by increasingly finer resolving techniques, can probably be listed in this group and have been shown to be modulated by the addition of auxin, apparently through their *de novo* synthesis. GLP while being necessary for the continued elongation, are not themselves part of the auxin primary response of the cell, as their increase does not occur until after cell enlargement has begun.

The changes in polypeptide synthesis, within the artichoke system utilised in this study, thought to be associated with the growth process, are those common to both the xylogenic and control cultures, as they both grow at the same rate, while only the xylogenic cultured explants produce xylem. (This makes the assumption that polypeptide synthesis associated with growth in artichoke explants is identical whether or not xylogenesis is concomitant.) The gap between the transfer of precultured explants (in which the explants do not grow) and the first assessment of protein

synthesis on either the xylogenic or the control medium (on which they do grow) is one day. As such therefore, it is not possible to note any primary changes occurring at the initiation of growth, however the general labelling trend throughout the culture periods can be commented upon. (It is interesting to note, as a cautionary remark, that the large increase in protein synthesis and the subsequent loss of polypeptides in the fluorographs, occurring during the preculture period happened during a time of absence in growth of the explants. Therefore the requirement for shorter time intervals to study the changes associated with explant growth and the limitations of the labelling studies over the long culture periods in this study are apparent.)

The changes in labelled polypeptides demonstrated in the one dimensional separation involves principally the loss of a 132 kdal polypeptide in both the particulate and cytosol fractions as growth declines and stops near the end of the culture period. The general trend over the culture periods in the two dimensional gels is one of reduction in protein synthesis, as evidenced by the reduced general radiospecificity of the separated polypeptides, coinciding with the reduction in growth of the explants. While many fluctuations in individual polypeptides have been noted in both the xylogenic and control cultures, very few of the 'arrowed' major changes are common to both cultures. However many minor and major polypeptides decrease in their labelling intensity over the culture period, indicating a general decrease in the various cellular processes within the plant cells, some of which will be integral to the growth process deceleration.

Xylogenesis within plant tissue culture or intact plants has not previously been studied at a high degree of resolution, with regard to protein synthesis. Utilising a one dimensional separation of ^{14}C-lysine labelled polypeptides and their location by fluorography, few changes were

noted throughout this cytodifferentiation process. Minor differences were noted between the xylogenic cultured and the control cultured explants, namely a fluctuation in a 20 kdal polypeptide and the presence of an extra 15 kdal polypeptide in the cytosol fraction of the control cultured explants. It was also noted that the radiospecificity of the proteins in the cytosol and particulate fractions of the control cultured explants was greater over the first two days of culture period than was apparent in the xylogenic cultured explants. This difference was enhanced by the much greater resolution offered by a two dimensional separation of the same proteins. An analysis of the two dimensional gels showed many changes in specific polypeptides throughout both the xylogenic and control periods, as well as many comparative differences in polypeptides labelled in the xylogenic and control cultured explants for any day of the respective culture periods. The differences were most marked, and hence analysed, within the first two days of the culture periods, as the high radiospecificity of the proteins allowed a detailed study to be undertaken.

There are many changes and comparative differences, over the first two days, within the xylogenic cultured explants, of which certainly some will be related to xylogenic initiation or development. In the cytosol fraction of the control cultured explants, many differences with the xylogenic culture exist. Some of the differences may be associated with a xylogenic inhibitory function, although probably the majority are associated with other tissue or cellular processes unrelated to xylogenesis.

Within the particulate located ^{14}C-lysine labelled proteins, while the detailed analysis of this fraction proved to be difficult, it was possible to see that on day 2 of the respective cultures there were many more polypeptides synthesised in the control cultured explants which were not present in the xylogenic cultured explants.

Given the general similarity of, at least, the initial preculture wound response, in terms of the polypeptide profiles, to the xylogenic cultured explants and the enhanced labelling of the proteins and the greater number of labelled polypeptides present in the control cultured explants, then there are indications that the xylogenic response of the artichoke tissue is a continuation of the initial wound response, while the control cultured explants follow a different non-xylogenic biochemical route. The positive influence of the preculture period on subsequent xylem element differentiation and the great similarity between the 'true' wound vessel members produced in wounded intact plants and the xylem elements formed in cultured plant tissues support these indications. In this sense, the initial wound response of the excised tissue would be continued and increased on transfer of the precultured explants onto the xylogenic medium, a situation in which the protein synthesis is almost channelled in one direction, while transfer to the control medium would open several avenues of cellular development causing, after a lag of one day, an increase in general protein synthesis and a wider production of individual polypeptides.

CHAPTER SEVEN

COVALENT MODIFICATIONS OF PROTEINS AND XYLOGENESIS

The turnover of key proteins will play a role in the determination and differentiation of cell types. The overall turnover of an enzyme or structural protein depends upon a balance between protein synthesis and degradation which can and does alter the activity levels of proteins within the cell. A further level of control has emerged in recent years, for specific proteins, in both plant and animal studies, that of the covalent modification of proteins after translation. This involves the covalent attachment of a specific chemical group to produce a modulated final product. Protein post-translational covalent modifications have been found to include; methylation, acetylation, thiolation, ribosylation, glycosylation, adenylation and phosphorylation. The two modifications studied in this report have been phosphorylation and glycosylation, in an attempt to correlate changes in protein modification with xylem differentiation.

Phosphorylation of Proteins

i) Phosphorylation of Enzymes

The phosphorylation and dephosphorylation of enzymes is known to be important in the coordination and control of intermediary metabolism (Cohen 1980), with the primary experiments on glycogen metabolism indicating

that phosphorylation is a major control point in this metabolic schedule. The list of enzymes so controlled has grown to twenty in animal studies (Krebs and Beavo 1979) while in plant studies only two enzymes; quinate:NAD oxidoreductase (QNO) (Refeno et al. 1982) and pyruvate dehydrogenase complex (PDH) (Rao and Randall 1980) have been reported to be reversibly modulated by phosphorylation modifications.

QNO catalyses the reversible conversion of quinate into dehydroquinate, leading into the shikimate pathway. It is active when phosphorylated, by endogenous protein kinase activity, and deactivated by endogenous protein phosphatase activity. PDH links the glycolytic pathway to the TCA cycle and is an important regulatory enzyme in both plants and animals. The regulation in plants by phosphorylation (inactivation) and dephosphorylation (activation) is as in animal systems. While only two examples of plant enzymic modulation by covalent modification have been reported, it would appear that it is a valid means of biochemical control in plants, which will probably yield further examples with further research.

ii) Phosphorylation of Chromatin associated proteins

The proteins, which together with DNA, compose the chromatin material, are histones (basic proteins) and non-histone proteins (NHP) (acidic proteins). Initially, in animal studies, histones were considered to be important in the role of gene regulators (Stein et al. 1974) but crude preparations of histones could only be fractionated into 5 classes, the proportions of which did not vary between tissues or in cells under varying physiological conditions. Histones do undergo a variety of post-translational covalent modifications, including phosphorylation e.g. the histone class, F_I, is invariably phosphorylated in dividing animal cells (Chalkeley et al. 1973) but the coupling of DNA synthesis and histone phosphorylation is not strong.

Acidic nuclear proteins have been shown to be heterogenous as well as demonstrating both tissue and species specificity (Allfrey et al. 1973). They have the ability to bind to DNA and can stimulate DNA dependent RNA synthesis in cell free systems. The synthesis and phosphorylation of specific NHP have been correlated with the induction of gene activity or cellular responses, at least one of which is the enzyme DNA dependent RNA polymerase (Jungman et al. 1974). For example in the slime mold, *Physarum polycephalus*, NHP have been shown to change, including phosphorylations, during differentiation (Le Stourgen and Rusch 1971). In HeLa cells 22 electrophoretically separated NHP bands have been established, certain of which underwent quantitative fluctuations throughout the cell cycle (Bourjee and Pederson 1972). The chemical induction of cancer in the liver of rats (Chiu et al. 1973) has indicated a role for NHP and their phosphorylated state in this cellular proliferation. A detailed study of protein kinases located in beef liver nuclei (Kish et al. 1974) further emphasised the tissue specificity of nuclear phosphoproteins and kinases, supporting the hypothesis of a role for NHP in gene regulation. The complexity and potential variability allowed by the array of kinases and NHP were considered to be of the order required for this, however there is as yet no unequivocal evidence that phosphorylation does control the putative regulatory nature of these proteins (Trewavas 1976).

The above examples are just a minute fraction of the reviews and papers associated with histone and NHP phosphorylation in animal studies and while plant research lags behind in this field, there have been recent detailed studies into plant nuclear protein phosphorylation.

Van Loon et al. (1975) studied the incorporation of ^{32}P into *Lemna* and barley nuclear proteins, followed by fractionation using acid-urea and SDS-electrophoresis. The *in vivo* studies indicated a complex profile of 15 to 20 phosphoproteins, with 80-90% of the label being in the NHP fraction.

In vitro analysis, using isolated nuclei, demonstrated endogenous protein kinase activity which, the authors suggested, retained a discrimination over the substrate to be phosphorylated, thereby being a suitable system for the *in vitro* analysis of chromatin protein phosphorylation. Further to this, Chapman et al. (1975) noted changes in chromatin protein phosphorylation during barley germination and while it could not be correlated with specific tissue differentiation it may have been associated with cellular differentiation (unknown or unspecified).

The most detailed analysis of plant nuclear protein phosphorylation has been by Murray et al. (1978) who analysed changes in this fraction in soybean, induced by the addition of 2,4-D. They determined that the increase in specific activity (phosphorylation) of the nuclear protein was cAMP-independent and could be correlated with the auxin induced increase in RNA synthesis, with the majority of the label being in the NHP. In the basic protein fraction, a low mobility non-histone basic protein demonstrated a substantial increase in phosphorylation after 2,4-D treatment, although the ^{32}P incorporation was small compared to the total incorporation. The authors expected the major changes to be associated with the chromatin attached NHP, and while changes were noted in this fraction, the most noticeable changes occurred in proteins easily removed from the nucleus. They suggested that these proteins may be involved in the packaging of mRNA and rRNA for export into the cytoplasm and that, in opposition to the general hypothesis i.e. nuclear protein phosphorylation causing the transcription of RNA, it would appear, in this study, that protein phosphorylation is a result of increased RNA synthesis.

Further to this report, Murray et al. (1978) isolated a chromatin associated protein kinase from soybean, which showed the biochemical features of a typical casein kinase found in plant and animal tissues; it phosphorylated casein, phosvitin and denatured chromatin proteins, but not

histones (a separate histone kinase has been isolated from soybean, Lin et al. (1976) and it is independent of cAMP. The role of the soybean casein kinase is unknown as it does not phosphorylate RNA polymerase I or II nor does it have any effect on *in vitro* chromatin directed RNA synthesis.

iii) Phosphorylation of Ribosomal proteins

The presence of phosphorylated ribosomal proteins in animal and plant systems has been well documented with ribosomes themselves demonstrating a weakly bound protein kinase activity. The majority of animal ribosomal kinases are cAMP dependent (Grankowski et al. 1974) while the equivalent plant kinases are cAMP insensitive.

Initially phosphorylation of ribosomal proteins was considered to be a potential regulatory mechanism for ribosomal activity in animal cells e.g. Kabat (1972) measured the turnover rate of phosphate groups in the reticulate located ribosomes and noted a difference between monosomes and polysomes. A regulatory role now appears unlikely, although the phosphorylated state of the ribosomal proteins may be associated with the maturation process of ribosomal development from the nucleolus to the cytoplasm (Olson et al. 1974).

Several groups have worked on plant ribosomal protein phosphorylation and their associated protein kinase activities. Phosphorylated ribosomal proteins have been demonstrated in *Lemna* (Trewavas 1973) from which a ribosomal protein kinase has also been isolated (Keates and Trewavas 1974). A similar tightly bound ribosomal protein kinase has been isolated from wheat embryo tissue (Carratu 1974) which displayed an ability to phosphorylate a variety of substrates, including endogenous proteins, and was insensitive to cAMP and cGMP.

Cytokinins have been shown to bind to a ribosomal protein (Fox and Erion 1975) and are thought to act, at least in part, through a modulation of ribosomal activity. Tepfer and Fosket (1975) undertook a detailed study of the effect of cytokinin on ribosomal protein phosphorylation utilising a cytokinin dependent soybean liquid suspension culture. A comparison of this culture with and without the addition of cytokinin demonstrated that monosomes and polysomes, in the cytokinin deprived cells, had a higher level of phosphorylation. Although no qualitative differences in ribosomal phosphoproteins existed between the two cultures, there were quantitative differences, with 3 of the 5 phosphoproteins being enhanced by up to two fold in proteins extracted from the cytokinin deprived cells. The authors concluded that, although one can correlate the activity of ribosomes to their state of protein phosphorylation, the evidence for this is not strong. The importance of ribosomal protein phosphorylation is therefore an open question and as Keates and Trewavas (1973) point out, the phosphorylation of ribosomal proteins may be a consequence of ribosome activation and not the cause of the activation.

Glycosylation of Proteins

Glycoproteins are important cellular constituents, being located in both the cytosol and the membranous fractions. The biochemistry and, often speculative, functions of glycoproteins have been reviewed by Brown and Kimmins (1977) and Sharon and Lis (1979).

Lamport has extracted a plant cell wall located glycoprotein, rich in the amino acid hydroxyproline, which he termed 'extensin' (Lamport 1965 and 1977). He speculated that it is associated in the wall with the control of wall extension during growth and while there is a certain degree of evidence in favour of this e.g. Cleland et al. (1967), others, notably

Winter (1971) and van Holst (1980) found no correlation between hydroxyproline incorporation into the wall and the growth of the tissue. The primary cell wall of higher plants may be seen to exist as a protein-glycan network in which proteins, pectins and xyloglucans serve to crosslink the cellulose microfibrils (Albersheim 1974). The glycoproteins would, in this manner serve a structural role although several enzyme activities have been demonstrated to exist in the wall fraction as well as hydroxyproline poor glycoproteins, the roles of which are unknown. (Selvendran 1975).

Recognition, at the cellular level, can be defined as specific cell to cell interactions capable of inducing a reaction(s) in consequence of this association (Heslop-Harrison 1978). Glycoproteins and lectins (specific carbohydrate binding proteins, themselves often glycoproteins) have been indicated in many cell-cell interactions. In Chlamydomonas species a glycoprotein, situated at the flagellar tip membranes, has been associated with the specific surface recognition occurring between mating partners at times of nutrient stress (Weise et al, 1978). Research into pollen-stigma compatibility interactions indicate that the pollen wall and stigma pellicle contain proteins or glycoproteins responsible for this specific interaction (Howlett et al, 1975 and Ghosh et al, 1980). Similarly, the molecules responsible for the phenomenon of acceptance or rejection between the graft and host in plants has been speculatively ascribed to proteins and possibly lectins (Yeoman et al. 1978). Specific surface glycoprotein components are required for the virulence of plant pathogens (reviewed by Albersheim and Anderson-Prouty 1975), as has been demonstrated to be the case for crown gall induction by Agrobacterium radiobacter in its host, and has also been shown to be of importance in the specificity of the interaction between the nitrogen-fixing, nodule forming bacteria, Rhizobium, and legumes (Bohlool and Schmidt 1974).

Glycoproteins have been strongly associated with cellular interactions in other organisms, including yeast mating (Crandell et al. 1978) slime mold development (Garrod et al 1978) and in animal embryogenesis, where endogenous lectins have been proposed to play an intercellular interacting role in development and tissue differentiation (Barondes 1981).

Of the many plant enzymes analysed, several have been demonstrated to be glycoproteins (glycoenzymes) eg. ricin from fig, a nuclease from mung bean and peroxidases, which are widely distributed throughout the plant kingdom and can be as much as 40% carbohydrate (Sharron and Lis 1979). The role of the carbohydrate moiety in this class of protein is unknown as the integrity of the oligosaccharide group is not a requirement for the activity of the enzyme eg mild periodate oxidation of bromelain (Yasida et al, 1971) causes 80% of the mannose residues to be destroyed, and most recently the inhibition of α amylase glycosylation with tunicamycin, both of which failed to effect the enzymic activity (Miyata and Akazara 1982). The saccharide portion of the glycoenzymes have therefore been tentatively ascribed a role as a 'tag' for enzyme recognition or siting within the cell.

Certain seed and plant storage proteins have been demonstrated to be glycoproteins, eg. glycoprotein I and II in kidney bean, legumin in garden pea and the 7s protein of soybean. The role of the carbohydrate moiety is again unknown and has been shown to be insignificant in, for instance, the complexing of the storage protein vicilin polymers into oligomeric complexes (Badenoch-Jones et al, 1981). The carbohydrate prosthetic group would appear to be significant in relation to water stress tolerance and the water imbibing properties of seeds during germination.

Protein glycosylation reactions usually involve a lipid linked intermediate (reviewed by Elbein 1979 and Lezica 1979) and, while the bulk of the research has been directed towards the elucidation of this system

in animal cells, it would appear that the plant protein glycosylation reactions are the same if not identical to those in animals. Studies indicate an "en bloc" transfer of the oligosaccharide, which is built up on the lipid dolichol, to the protein, which on glycosylation can be further processed by 'trimming reactions' utilising membrane bound specific glycosidases to produce the final product. The use of metabolic inhibitors such as; tunicamycin, amphomycin, GDP-deoxyglucose and fluoroglucose have greatly helped in the determination of the associated metabolism of protein glycosylation (Schwartz and Datem 1980). Several questions relating to plant protein glycosylation remain to be answered; the exact nature of the lipid intermediate, the subcellular locations and the regulation of the glycosylation reactions, the processing of the pre-product and the role or functions of the products. The rough endoplasmic reticulum is the major site of protein glycosylation and the golgi apparatus is a minor site of protein glycosylation (Nagashi and Beavers 1978 and Mellor and Lord 1979), the membranous environment of these sites prossibly explaining the importance of the lipid nature of the intermediate, dolichol, in protein glycosylation.

In summary, although many glycoproteins have now been determined in plant tissues, there is as yet no conclusive evidence as to the role played by the carbohydrate moiety in the cellular function of the glycoprotein. However, it appears highly unlikely that a complex series of glycosylation reactions, involved in the production of a glycoprotein, would have been developed or conserved, if the carbohydrate group did not play a definite role in the metabolism or recognition of these components.

The analysis of protein covalent modifications undergone in a differentiating plant cell has not been previously studied. Tunicamycin, an inhibitor of protein glycosylation, has been shown to inhibit the differentiation of xylem elements in cultured artichoke tissues (chapter

5) indicating the possible importance of protein glycosylation in the xylogenic process. Protein phosphorylation has been demonstrated to modulate enzyme activities and also possibly to modulate gene activity and organelle (microtubule and ribosomal) activities, all of which could possibly be significant within the xylem cytodifferentiation sequence. A study of protein glycosylation and phosphorylation was therefore undertaken in an attempt to determine if these specific covalent changes could be correlated with the xylogenic process.

RESULTS

a) ^{14}C-Fructose Incorporation

^{14}C-Fructose was used as a 'tag' for studying changes in the glycosylation of proteins as evidenced by the fluorographic representation of the separated polypeptides.

Figure 7.1 shows the fluorographs for the cytosol fraction of the cultured explants. In the preculture period very little incorporation is again found on day 0, but more bands are apparent than with the ^{14}C-lysine studies. Band f, MWr 21 kdal, increases over the preculture period and remains fairly constant in subsequent cultures, while band g, MWr 24 kdal, appears on day 1 of the preculture period but disappears after this. When comparing the band intensities of polypeptides separated from the xylogenic and control cultures, several differences can be noted. Bands a, MWr 14 kdal, and b, MWr 15 kdal, while diminishing throughout the xylogenic period, disappear completely after one day in the control culture. Band c, MWr 19 kdal, reduces more so during the xylogenic period when compared to the control, while band d, MWr 44 kdal, disappears over the first two days of the xylogenic culture period, but remains constant throughout the control and preculture periods. Band e, MWr 77 kdal, similarly disappears over days 3 and 4 of the xylogenic period, reappearing on day 5. This polypeptide remains constant in the other two cultures.

The ^{14}C-fructose labelled polypeptides separated from the particulate fraction are easier to analyse, when compared to the lysine studies, as the 'background' is not as intense. The only noticeable change (Figure 7.2) in the preculture incorporation studies, other than the low incorporation on day 0, is that of band h, MWr 14.5 kdal, which increases over this period. On comparison of the xylogenic to the control cultures,

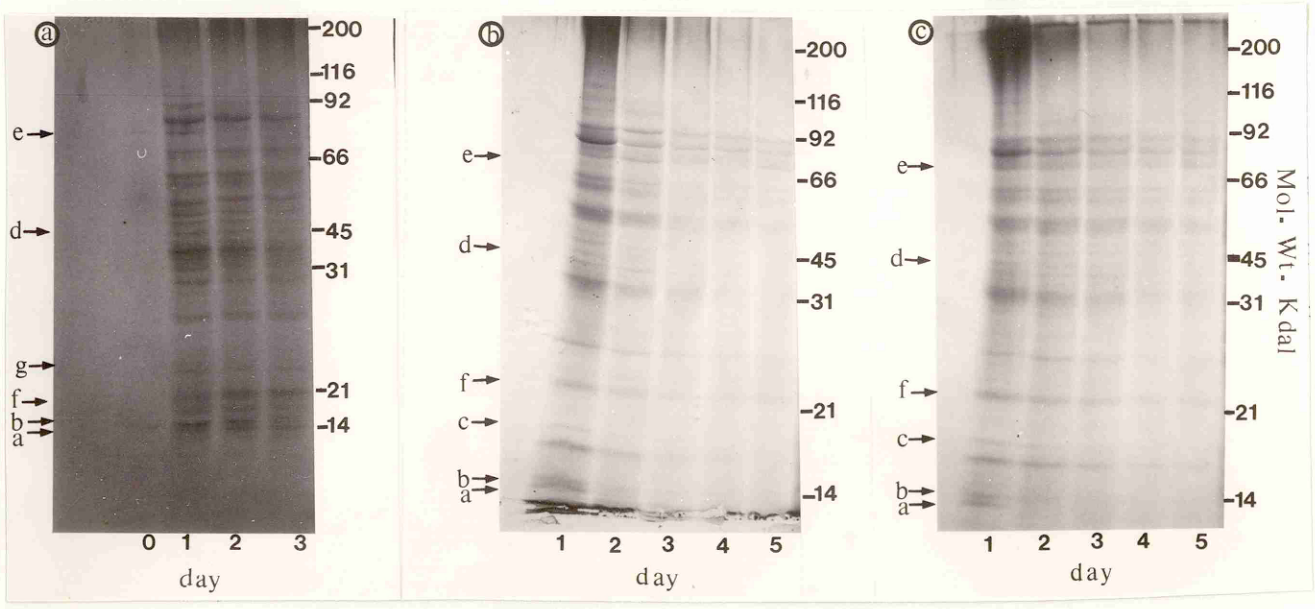

Figure 7.1 Fluorographs of the artichoke homogenate cytosol located, ^{14}C-fructose labelled proteins, separated by SDS-PAGE for each day of (a) preculture, (b) xylogenic culture and (c) control culture.

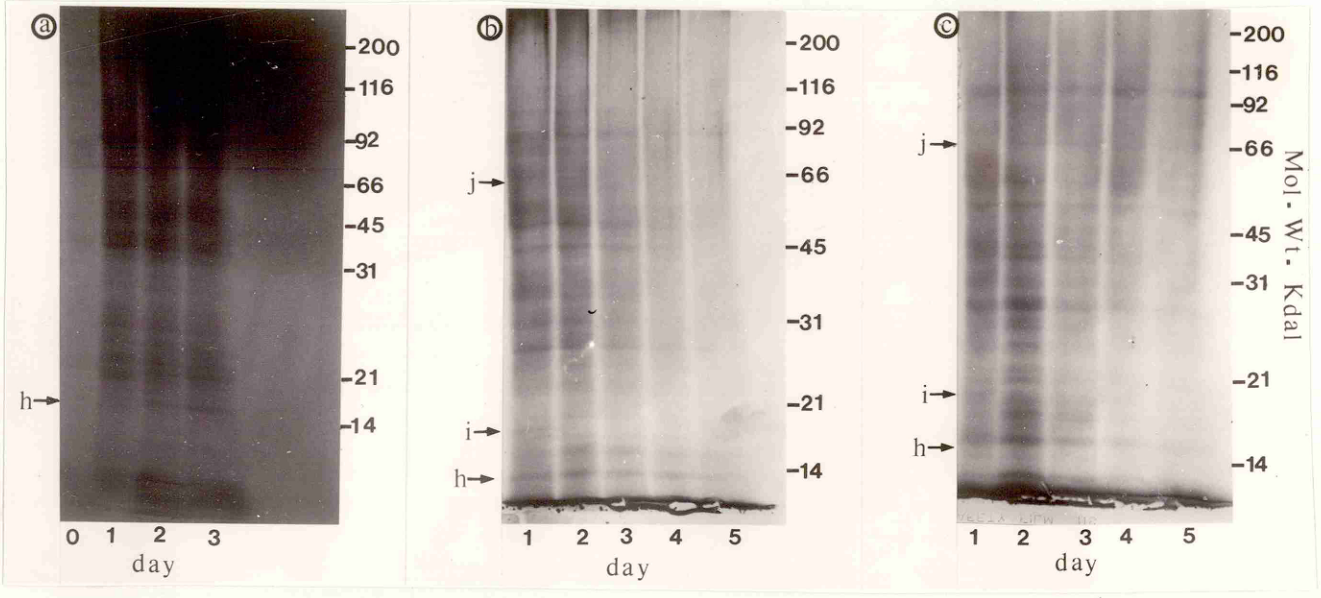

Figure 7.2 Fluorographs of the artichoke homogenate particulate located, ^{14}C-fructose labelled proteins, separated by SDS-PAGE for each day of (a) preculture, (b) xylogenic culture and (c) control culture.

band i, MWr 18 kdal, disappears after day 1 in the xylogenic culture, while band j, MWr 64 kdal, only appears after day 1 in the control cultured explants.

b) ^{32}P-Phosphate Incorporation

The autoradiographs of separated cytosol located polypeptides showed that only relatively few phosphorylated bands are present in this fraction (Figure 7.3). During the preculture period there was no ^{32}P-phosphate incorporation on day 0, while band d, MWr 57 kdal, appeared only on day 1. Bands a, MWr 30 kdal, and b, MWr 105 kdal, remained constant throughout this period. In the control culture, band b remains constant while band a is relatively intense on day 1 but diminishes after this. An extra band, marked c, MWr 35 kdal, is apparent on day 1 of the control culture only. In explants undergoing xylogenesis bands a and b undergo similar fluctuations to that of the control cultured explants. Several other striking changes can be noted; band e, MWr 89 kdal, is apparent on days 1 and 3 but disappears on day 2 of the xylogenic period, while band g, MWr 200 kdal, increases markedly in intensity on day 3 of this period. Most noticeably, upto 8 extra phosphorylated polypeptide bands appear on day 3 of the xylogenic period, marked f, with a MWr range from 84 kdal to 220 kdal.

In the autoradiographs of the ^{32}P labelled particulate fractions (Figure 7.4) the 'background' again makes analysis difficult. There would appear to be a greater phosphorylation of proteins in this fraction or possibly there is interference due to the presence of labelled phospholipids. During the preculture period little change is noticeable while in the xylogenic and control cultures band a, MWr 30 kdal, also present in the cytosol, increases in intensity on day 1 diminishing over the remaining period. It is interesting to note that on day 3 of the xylogenic culture

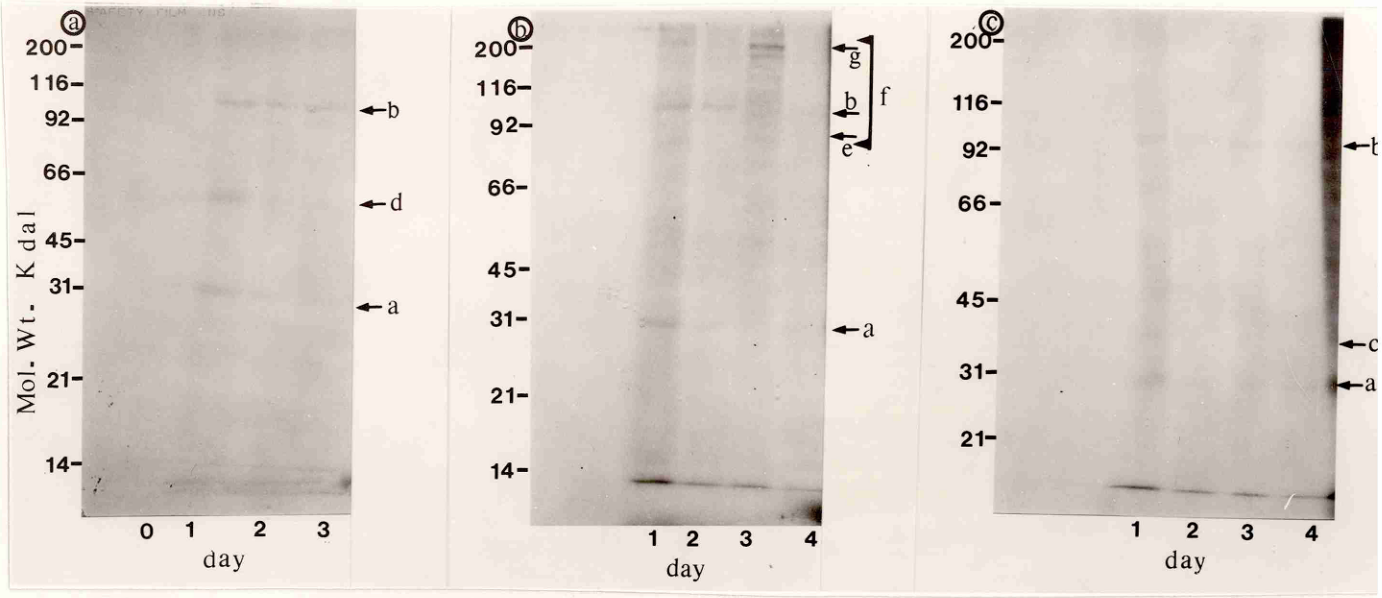

Figure 7.3 Autoradiographs of the artichoke homogenate cytosol located, ^{32}P-phosphate labelled proteins, separated by SDS-PAGE for each day of (a) preculture, (b) xylogenic culture and (c) control culture.

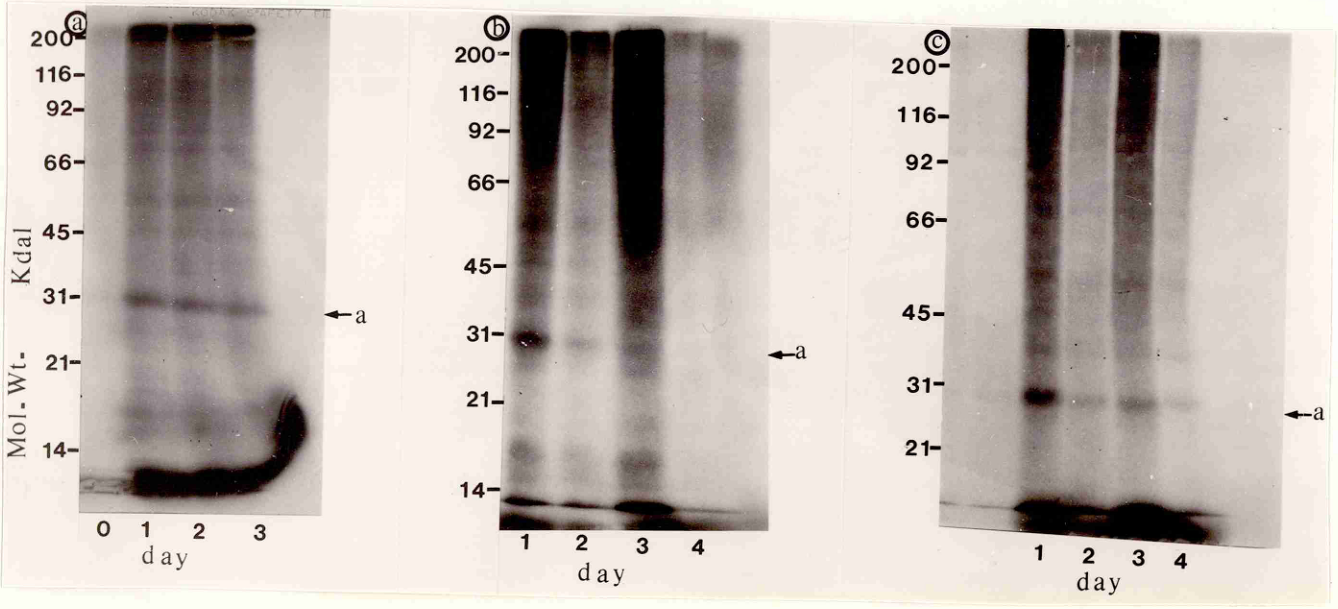

Figure 7.4 Autoradiographs of the artichoke homogenate particulate located, ^{32}P-phosphate labelled proteins, separated by SDS-PAGE for each day of (a) preculture, (b) xylogenic culture and (c) control culture.

the region of intense ^{32}P location, by autoradiography, corresponds to the region of new phosphorylated polypeptides in the cytosol fraction.

The peak of xylem differentiation occurred on day 3 of the culture used in the phosphate labelling experiment. The photographs are representative of two separate labelling experiments and several gels from each experiment.

c) Protein Kinase Studies

Protein phosphorylation was seen to be the most marked change during xylem differentiation in cultured artichoke explants. A preliminary investigation was undertaken to determine if plant hormones had a direct effect on the protein kinase activity present in the culture tissues. Protein kinase activity was measured as the ability, of the cytosol and particulate fractions from the artichoke homogenate, to phosphorylate an exogenous protein source. (Casein has been utilised as the protein substrate in the majority of other plant preliminary studies and was therefore used in this study also). Protein phosphorylation was quantified as pmol of ^{32}P incorporated per mg of casein present in the kinase assay.

The time course of the protein kinase activity was determined by allowing the assay period to be extended (Figure 7.5). As can be seen from this graph, the incorporation of ^{32}P into casein is maximal after 10 minutes, beyond which time the incorporation declines. (This reduction with time is probably due to the presence of endogenous dephosphorylation activities in the fractions). A similar graph was obtained using the particulate fraction and a 10 minute assay period was used in further protein kinase assays.

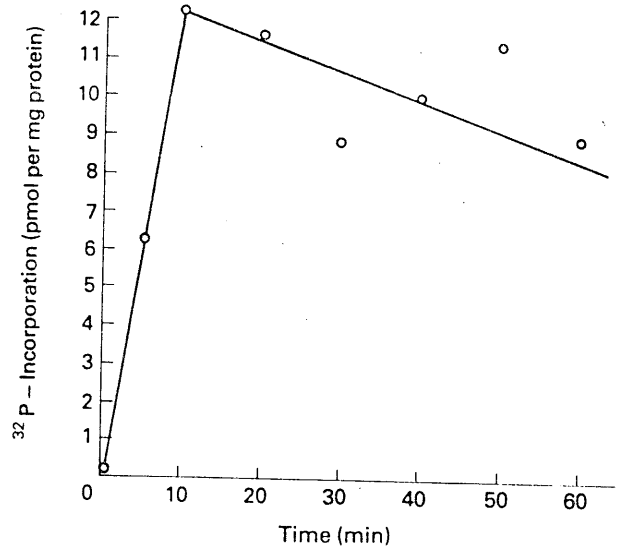

Figure 7.5 The time course of casein kinase activity in the cytosol fraction of artichoke explants cultured on the preculture for 3 days. Each point is the mean of 3 separated assays.

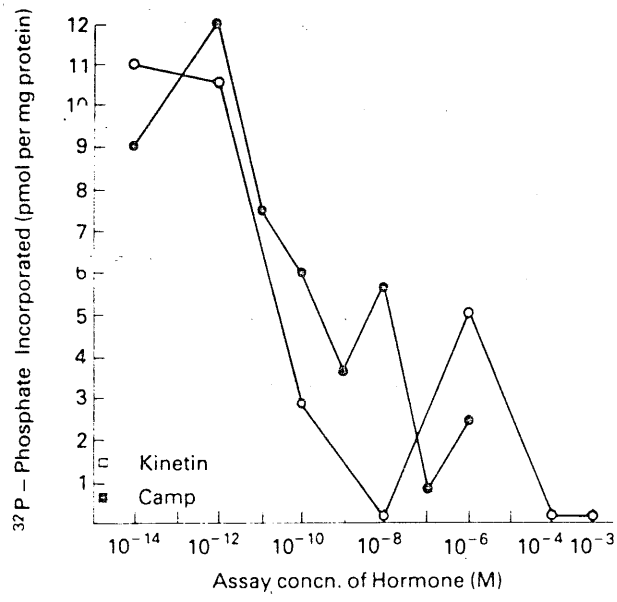

Figure 7.6 The modulation of *in vitro* casein kinase activity, located in the cytosol fraction of artichoke explants, on the addition of cAMP (closed circles) and kinetin (open circles). Control casein kinase activities (no addition of exogenous hormone) were determined for the cAMP experiment (closed square) and the kinetin experiment (open square). The explants were cultured on the preculture medium for 3 days prior to homogenisation and fractionation. Each point is the mean of three assays.

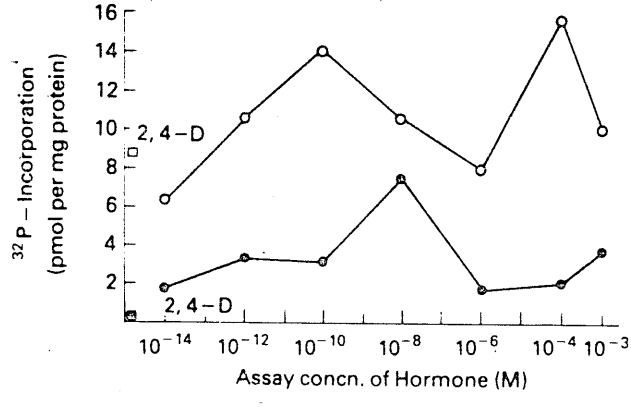

Figure 7.7 The modulation of *in vitro* casein kinase activity on the addition of 2,4-D to the particulate fraction (open circles) and cytosol fraction (closed circles) of artichoke explants. Control casein kinase activities were determined for the particulate fraction (open square) and the cytosol fraction (closed square). The explants were cultured on the preculture medium for 3 days prior to homogenisation and fractionation. Each point is the mean of three assays.

To determine the effect of plant hormones and cAMP on the casein kinase activity, various concentrations of the above chemicals were added to the assay medium. Figures 7.6 and 7.7 are representative graphs from several repeats of each experiment. Figure 7.6 demonstrates that, on the addition of cAMP, there is a significant increase in the casein kinase activity in the cytosol fraction, peaking at an assay concentration of 10^{-12} M cAMP. A very similar concentration/activity profile was observed on the addition of equivalent concentrations of kinetin to separate assays, with maximum activity again at 10^{-14} to 10^{-12} M kinetin. Note that the casein kinase activity is negligible in the absence of the added hormone or cAMP (the open and closed squares in Figure 7.6). At least two further reproducable peaks of activity were observed in figure 7.6, probably reflecting the presence of more than one protein kinase in the fraction. The particulate fraction of the artichoke explant homogenate, while containing upto 10 times the casein kinase activity witnessed in the cytosol fraction, gave no pattern of kinase modulation upon addition of cAMP or kinetin.

Figure 7.7 shows the effects of the synthetic auxin, 2,4-D, on casein kinase activity located in cultured artichoke explants. This graph differs in several ways from that shown in figure 7.6. Firstly the modulation of casein kinase activity by the hormone is observed in both the cytosol and the particulate fraction. Secondly, it can be seen that the level of kinase activity is relatively high, in the absence of auxin, in the particulate fraction when compared to the cytosol fraction. Thirdly, while with cAMP and kinetin the maximum kinase activity is apparent at concentrations of 10^{-14} to 10^{-12} M kinetin/cAMP, the casein kinase activity is minimal for the equivalent concentration of 2,4-D. Casein kinase activity peaks, in the particulate fraction at 10^{-10} and 10^{-4} M 2,4-D, and in the cytosol fraction at 10^{-8} M 2,4-D.

DISCUSSION

Protein synthesis has been shown, both previously and in this study, to be important for the differentiation of xylem in plant tissues. To this end a detailed analysis of the polypeptides synthesised in artichoke explants during the xylogenic process has been undertaken (chapter 6). To complement this preliminary investigation, a relatively simple approach to the determination of protein covalent modifications (glycosylation and phosphorylation) was also undertaken in an attempt to correlate observed changes in the covalent modification of proteins with xylem differentiation.

In a similar manner to the discussion of the ^{14}C-lysine studies, the covalent modifications will be grouped in an attempt to correlate changes either with the wound response, the growth response or with xylem differentiation in the cultured artichoke tissues. As in the radiolabelled amino acid incorporation studies, there is very little ^{14}C-fructose or ^{32}P-phosphate incorporation on day 0 of the preculture period, due to the dormancy of the tuber tissue, after which time a large number of ^{14}C-fructose labelled polypeptides and relatively few distinct phosphorylated bands were apparent throughout the remaining culture period and the subsequent xylogenic and control cultures. During the preculture period little change occurred in the ^{14}C-fructose and ^{32}P labelled polypeptide profiles except for a 24 kdal glycosylated polypeptide and a 57 kdal phosphorylated polypeptide, both of which only appeared on day 1 of the preculture period and were probably associated with the excision wound response of the cultured tissue.

Changes in the modified polypeptide profiles thought to be associated with the growth of the explants (ie. common to both the xylogenic and the control cultures) were relatively few in the glycosylated and phosphorylated patterns, however, a 30 kdal phosphorylated polypeptide was noticeable in

both the control and xylogenic cultures, being intensely labelled on day 1 of these cultures after which its intensity declined as the growth rate declined. This band was also present in the precultured explants but its intensity of labelling remained constant throughout the preculture period.

A comparison of the glycosylated polypeptide profiles throughout the xylogenic and control cultured periods showed more changes than in the one dimensional radiolabelled amino acid study; a 19 kdal and 44 kdal glycosylated polypeptide disappeared gradually from the cytosol fraction and an 19 kdal glycosylated polypeptide after day 1 in the particulate fraction during xylem differentiation. In the control cultured explants a 14 and 15 kdal polypeptide in the cytosol disappeared after 1 day and a 65 kdal glycosylated polypeptide appears in the particulate fraction after day 1. While analysis of the glycosylated proteins located in the particulate fraction was clearer than for the ^{14}C-lysine and phosphorylated polypeptides located in the particulate fraction, few changes were noted, at the resolution offered by a 1 dimensional analysis, while a two dimensional analysis of the glycosylated polypeptides would possibly have resolved further masked changes.

^{14}C-Fructose was utilised as a tag for glycoproteins present in the artichoke explants during culture. The radiolabelled profiles formed using a radiolabelled sugar will depend upon several factors; the biochemical interconversions of the radiolabelled precursor into other intermediates, the constitution of the glycoprotein oligosaccharide moieties and also whether in fact glycosylation is a regulatory modulation associated with wounding, growth or xylem differentiation. The alterations observed in the fluorographs would appear to be actual glycosylations or deglycosylations as the equivalent molecular weight bands are relatively static in the ^{14}C-lysine labelled polypeptides. The significance of the changes are unknown

in this preliminary investigation, but may play a role in the intracellular location of the proteins or modulate the polypeptide (protein) interaction(s) with other cellular constituents, especially if they posses lectin-like properties, both of which could be significant within the xylogenic process.

By far the most marked changes in labelled polypeptide patterns occurred in the phosphorylated polypeptide profiles in which several bands, of MWr from 84 to 220 kdal, appear in the cytosol on day 3 of the xylogenic culture, at the same time as the maximum rate of xylem differentiation, while being absent in the control cultured explants. Protein phosphorylation is recognised as a major mechanism by which intracellular events respond to external physiological stimulii. While the majority of work has involved animal studies an increasing body of information indicates that protein phosphorylation is of importance in plants also. (Reviewed by Trewavas 1976). Phosphorylation studies can be grouped into those relating to; enzyme activity modulations, chromosomal protein phosphorylation (and possible gene activiation), organelle phosphorylation (ribosomes and microtubules), the protein kinase molecules themselves and their activation by secondary messengers, such as cAMP. In the protein phosphorylation studies discussed above the polypeptide phosphorylation changes occurred at the same time as the maximum rate of xylem differentiation, which is assessed by the appearance of the characteristic secondary wall of the xylem elements. The final sequence of the xylem cytodifferentiation sequence is that of autolysis, when the living cell contents of the xylem element is removed by a variety of enzymic activities and it would appear probable that, at least, certain of the protein phosphorylations demonstrated may be related to the activation of enzymes involved in the final autolytic phase of xylem differentiation.

Proteins are phosphorylated by protein kinases and while there is relatively little known about plant protein kinases, those that have been studied are cyclic nucleotide independent and show a substrate preference for acidic proteins, such as casein and phosvitin, being very similar to animal cyclic nucleotide independent protein kinases. Keates (1973) studied protein kinase activity in the cytosol fraction of homogenised pea shoots and found, using column chromatography, 4 kinase peaks with varying substrate preferences but all insensitive to the addition of cAMP. Similarly Carratu (1974) using wheat embryos, found the post-ribosomal supernatant to contain 3 kinase peaks with similar properties to that described by Keats. A more detailed analysis of two post-ribosomal protein kinases with a substrate preference for casein (casein kinase I and II (CKI and CKII)) isolated from soybean cotyledons was carried out by Gowda and Pillay (1982). On purification with columnchromatography and analysis by SDS-PAGE, CKI was shown to be a single subunit protein of MWr 39 kdal, while CKII was multimeric with 3 subunits of MWr 52, 37 and 35 kdal. CKII phosphorylated 40s ribosome subunits and their associated proteins, while CKI and II underwent autophosphorylation, a phenomenon also shown to exist in animals (Hathaway and Traugh 1979). Autophosphorylation may be a general modulatory mechanism for cyclic nucleotide independent protein kinases.

The widespread distribution of cyclic nucleotide mediated processes in animals and certain micro-organisms (one of which is to modulate protein kinase activity) initiated a large scale search for similar responses in plant tissues. Initial research produced variable and often conflicting data (reviewed by Amrhein 1974 and Lin 1974) with the general conclusions that either cAMP did not exist in plants or that the level is very low, thereby making the animal role as a secondary messenger unlikely in plant systems. More recent reviews by Amrhein (1977) and Trewavas (1975) have not removed the major objections raised in the earlier reviews although

much research still continues in this field. One of the problems with the study of cAMP in plant tissues has been the presence of 'interferring substances' peculiar to plant extracts which interfere with the quantification of cAMP, (Brown et al 1979). The authors suggested that with adequate purification of the plant extracts and the use of internal standards the presence of cAMP, adenylate cyclase activity and cAMP binding protein activity can all be determined in Phaseolus vulgaris extracts.

An exacting study by Ashton and Polya (1977 and 1978) demonstrated cAMP to be present in Kalanchoe daigremontiana, Agave american and rye grass at concentrations of 2 to 6, 1 and 2 to 12 pmol per gm fresh weight respectively. The authors calculated that this would be a cytoplasmic concentration of 0.2 μM, comparable with animal studies. They suggested that the interactions between cAMP and a "multiplicity of proteins" in higher plants are functional rather than fortuitous. Contrary to the above, Hintermann and Parish (1979) attempted to determine adenylate cyclase (the enzyme responsible for the production of cAMP) activity in a variety of organisms. This activity was found in a blue green alga, several green algae, slime molds, a fungus and a moss protenema, but not in any of the higher plant tissues studies. The presence of this activity in lower plants and not in higher plants is certainly a puzzle.

cAMP frequently acts through its specific binding to a protein receptor which is, for instance, associated with a protein kinase molecule as a regulatory subunit. A cAMP binding protein (cABP) was originally discovered in Jerusalem artichoke tuber and analysed in detail by Giannattasio et al (1974 and 1979). The protein has been purified to electrophoretic homogeneity, with a molecular weight of 240 kdal and a K_d for cAMP of 2.3×10^{-7} M. It exhibits two binding sites; one for cAMP and adenosine, the other for adenosine only, however, no physiological role has

been determined for this factor as it fails to exhibit kinase activity or the activation of DNA dependent RNA synthesis. Two cABP's have also been observed in wheat germ (Polya and Bowman 1981), one with a K_d for cAMP of 10^{-7}M and the other of 10^{-6}M and both with a marked adenine analogue specificity (including adenine derivatives with cytokinin activity), again the physiological significance of the protein is unknown. While the evidence for cAMP in plants mounts, there must still be doubts retained until the varying findings can be united to equate with the system determined for animals or a system is hypothesised to account for the presence of cAMP in plants in a different role to that in animals.

In the studies accounted for in this report protein phosphorylation would appear to be relevant to the process of xylem differentiation, and therefore a preliminary investigation into the endogeneous protein kinase activity of artichoke tuber tissue was undertaken. Interestingly cAMP was found to activate casein kinase in the cytosol fraction of the homogenate, however a very similar kinase activity/concentration profile was determined when the cytokinin, kinetin, was used in the assay to determine protein kinase activity. This similarity probably reflects the structural likeness of the two molecules. Ralph et al (1972) studying general protein phosphorylation in chinese cabbage discs, noted a stimulation of protein phosphorylation (in vivo) on the addition of kinetin, while IAA, cAMP and GA did not affect protein kinase activity. They tentatively concluded that in plants cytokinins replaced the role of cAMP in animals and also referred to the fact that hexokinase has been shown to be directly modulated by 6-benzylaminopurine, suggesting that it may also act directly on protein kinases. The similarity of cAMP to cytokinins has also been pointed out by Amrhein (1977) who states that it would be very difficult to distinguish between cAMP and cytokinin mediated responses in plants, which would appear to be the case in this study also. The maximal casein kinase activity occurred at a concentration of cAMP/kinetin of 10^{-14}

to 10^{-12}M, a level much too low for either to act as secondary amplification messenger, cAMP must be said to be acting as a cytokinin in this study.

Auxin (2,4-D) was also seen to directly modulate protein kinase activity, but it differred from cAMP/kinetin activation of casein kinase in that the casein kinase/hormone concentration profile was seen to be very different for 2,4-D and the auxin mediated protein kinase activity was located in both the cytosol and the particulate fraction of the artichoke homogenate. In both the studies with auxin and cytokinin mediated protein kinase activation, it would appear that the actual activity profiles are an amalgamation of a population of kinases present in the crudely fractionated cytosol and particulate fractions and would require further fractionation by column chromatography prior to a more detailed analysis.

In summary, although a cAMP enhanced protein kinase has been determined in artichoke explants, it would appear that cAMP is acting as a cytokinin in its modulation of the casein kinase activity. Trewavas (1976) does not see the requirement for an animal "cAMP-type" system in plants in view of the fact that plant hormones can enter the cells more readily than certainly, the proteinaceous animal hormones and therefore need not require a secondary messenger, with the primary message directly modulating the various cellular processes. In view of the possible correlation between xylem differentiation, protein phosphorylation and the ability of auxins and cytokinins to directly modulate the *in vitro* protein kinase activity in artichoke, then a possible hypothesis for the direct plant hormone initiation or control of xylem element differentiation, acting, at least in part, through protein plant kinases and the phosphorylation of certain key proteins, is an intriguing possibility.

CHAPTER EIGHT

SUMMARY AND GENERAL DISCUSSION

The primary intention of this project was to study some biochemical aspects of xylem differentiation. General observation indicated that this study would be best approached by the utilisation of plant tissue culture techniques, which allows for an element of experimental control (compared to xylem differentiation in the intact plant) and in which xylem element formation occurs rapidly. While several culture systems were available for this study, none fully met the criteria necessary for the detailed biochemical investigation of this cytodifferentiation process and it was therefore decided to modify a pre-existing artichoke system to this end.

The novel culture regime appears to meet the requirements for the biochemical study of xylem differentiation, in that a large number of xylem elements are formed over a short culture period.

A two-step culture regime was originated, in which Jerusalem artichoke explants were firstly cultured on a preculture medium, during which time it was possible to determine infection of the explants and hence preclude these from further experiments. During the preculture period the explants did not grow or produce xylem elements, although from the various investigations undertaken it would appear that biochemical events occurring

during this period are significant to the subsequent xylem formation. Following preculture the uninfected explants were transferred to either a xylogenic medium (on which tissue growth and xylem differentiation occurred) or a control medium (on which the explants grew at the same rate as the xylogenic cultured explants but without significant xylem formation).

The starting tissue is relatively simple, as is the culture technique involved and the use of the precuiture, xylogenic and control culture media should enable the separation of the biochemical events associated with the excision wound response, growth response and xylogenic response of the cultured tissues.

Hormone combinations (auxin and cytokinin), and especially the level of auxin, have been shown to be important in the induction of xylem element formation in cultured artichoke explants. An auxin-binding protein had recently been found in cultured artichoke tissues and therefore it was decided to investigate this phenomenon further in an attempt to correlate changes in such a factor with the xylogenic process. A cytosol located auxin (in this case 2,4-D) binding protein was determined in the cultured artichoke explants, very similar to the one previously found, with a more detailed analysis of the kinetics and specificity of hormone binding of this factor being undertaken.

In addition a similar 2,4-D binding protein was also observed in the artichoke particulate fraction, something not previously found, being similar in most respects to that located in the cytosol fraction with the exception that IAA failed to compete with 2,4-D for the binding site. While this is a puzzling observation, it is not without precedents, as a similar failure of IAA to displace phenoxyacetic acid derivatives from a particulate located auxin binding protein has also been established in pea.

The tissue concentration of the 2,4-D binding protein was observed to fluctuate in a characteristic and reproducible manner on transfer of the artichoke explants to the various media, but there was no correlation observed between this fluctuation and xylem differentiation or indeed with the growth of the tissues. These fluctuations may reflect some aspect of the tissues wound response and/or a reaction by the tissues to being transferred to a new medium; a subculture response.

An analysis of the proteins present in the cultured artichoke tissue, throughout cytodifferentiation, was similarly thought to be a fruitful avenue of investigation, as any changes observed would be a preliminary analysis of changes in, for instance, enzyme levels or structural proteins, both of which are probably significant to the xylogenic process. Inhibitor studies indicated the importance of protein synthesis (as well as mRNA synthesis and protein glycosylation) to the differentiation of xylem elements, however a simple analysis of the proteins present in the tissues, utilising gel electrophoresis and coomassie blue staining, demonstrated an absence of change during xylem differentiation. A more detailed investigation of protein synthesis, using a radioactively labelled precursor amino acid and a one dimensional separation of the labelled polypeptides by SDS-gel electrophoresis, showed a few minor changes thought to be associated with xylogenesis, growth or wounding respectively. A two dimensional analysis of the labelled polypeptides indicated a much greater number of differences between the comparable control and xylogenic cultures, some of which will probably be directly associated with xylem differentiation. A general comparison of the trends in polypeptide synthesis during either xylem differentiation or simple growth, indicated that xylogenesis was, in many respects, an apparent continuation of the initial wound response of the cultured tissues, while growth in the control culture allowed for a greater diversity of polypeptide synthesis, particularly in the particulate fraction, indicating an 'opening out'

of the synthetic potential of the tissues for further and less restricted differentiation.

Utilising similar techniques (radiolabelled precursor incorporation, electrophoresis and auto/fluorography) an analysis was undertaken into the possible role post-translational covalent modification of proteins plays in the initiation or control of xylem differentiation. While a few glycosylation protein modifications were noted, by far the most noticeable changes occurred in the cytosol located phosphoprotein fraction, where several polypeptides were phosphorylated at the time of peak xylem differentiation. Several organelles or organelle constituents and enzymes are known to modulate by reversible phosphorylation and it is thought that the protein phosphorylations noted in this study may be significant in the control of some aspect of the xylem differentiation sequence, for example the activation of cytosol located enzymes responsible for the autolytic phase of the xylogenic sequence.

Given the apparent significance of protein phosphorylation to the differentiation of xylem in cultured artichoke tissues, a further study into the direct control of protein kinase activity, by plant hormones and cAMP, in artichoke was carried out. Protein kinase activity (as measured by the *in vitro* incorporation of ^{32}P into casein) was observed in both the particulate and cytosol fraction of artichoke tissue. cAMP was found to modulate the acitivity the protein kinase activity in the cytosol fraction, however this activity profile was reproduced when the cyclic nucleotide was replaced by a cytokinin, kinetin. In view of the structural similarity of the two chemicals and the very similar activity profiles, it is suggested from these studies that cAMP is acting as a cytokinin in the activation of protein kinase in artichoke tissues. A synthetic auxin, 2,4-D, was also shown to modulate protein kinase activity but at a different concentration to that of cAMP and kinetin and in the

particulate as well as the cytosol fractions of the artichoke homogenate. There is the possibility of a direct hormonal control of xylem element differentiation in plant tissues, acting, at least in part, through the modulation of protein kinase activity and the phosphorylation of possible key proteins associated with the differentiation process.

With regard to future research, an obvious line of investigation is to enlarge upon the rather preliminary findings of the association between xylem element formation and protein phosphorylation. Initially this would involve the optimisation of the *in vivo* phosphorylation conditions to obtain the most efficient results to be further analysed by electrophoresis and autoradiography. Secondly a more favourable resolution of the particulate located phosphoproteins is required to obtain the maximum information possible from the autoradiographs. This will involve either the further fractionation of the particulate fraction by, for example, gradient centrifugation, into organelles and other membrane fractions, with the additional experimental and analytical hazards this entails, or the delipidation of the particulate fraction to prevent the apparent interference by the lipid fraction.

A more detailed investigation is also required of the protein kinase activity observed in the artichoke homogenate, which is thought to be probably a population of kinases in the crude extracts. This would involve the separation of the variously located protein kinases by, for example, gel chromatography, followed by an analysis of each fraction in terms of substrate specificity and hormone or secondary messenger specificity as a modulator of the kinase activity.

Ultimately it would be hoped that at least certain of the polypeptides phosphorylated during xylem differentiation could be identified, possibly as an enzyme. This could be approached by the fractionation of the

phosphoproteins under non-denaturing conditions followed by an analysis of the phosphoprotein fractions for enzyme activity or the investigation of enzymes thought likely to be significant in the cytodifferentiation sequence and determining if it is modulated by reversible phosphorylation. While both of these approaches are rather hit and miss, important advances could be made in the understanding of the control within plant cellular differentiation at the biochemical level.

It is difficult to draw any truly general conclusions from the findings reported in this thesis, in view of the nature of the work which is, in many respects, a preliminary ground work for potential future research. The culture regime described and discussed in this report will be as useful as the results and findings which can be obtained in its usage towards an insight into xylem differentiation growth, wounding, or, in fact, all three if they are integrally related.

An interesting facet of the xylogenic induction studies is the apparent prominence of the preculture phase within the overall xylogenic process. While more research is required, to determine the actual relationship between the two halves of the xylogenic culture regime utilised in these studies, it is possible to at least tentatively hypothesise that the initial phase enhances the level of xylem differentiation by 'priming' a significant proportion of the artichoke cells for subsequent xylogenesis. This priming maybe associated with a synchronisation of the cells at, for instance, a specific phase of the cell cycle (in view of the absence of cell division during this phase), or in either a specific or general biochemical readiness for xylogenic cytodifferentiation (possibly as an extended and exaggerated wound response).

With regard to the work relating to plant hormone induction of cytodifferentiation, hormone binding, protein kinase activity, and protein

phosphorylation, while relatively little progress has been made as to their coordination within xylem differentiation, if indeed they are, sufficient progress has been made to indicate the possible importance of at least certain of these biochemical happenings within cytodifferentiation and even possibly general plant hormone control. The artichoke culture system allows for a means of looking into this further, enlarging upon the preliminary indications to hopefully gain a more detailed insight into the plant hormonal control of cellular biochemistry and cytodifferentiation.

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